A STUDY OF THE LEUCINELESS MUTANTS OF NEUROSPORA CRASSA

Thesis

by

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INTRODUCTION

In 1941 when Beadle and Tatum published the first of the papers dealing with the biochemically delineated mutant strains of Neurospora it was appreciated that a new method had been derived for the study of the genetic control of biochemical reactions. Through the efforts of these and other workers Neurospora has since taken its place with those organisms adaptable to the study of genetic problems in terms of biochemical reactions. Not only have the original expectations been realized, but also many ramifications of the problem have arisen. Briefly stated the fundamental problem has sought an answer to the question, "How do genes regulate the functions of living organisms?"

The stimulus for the approach used was provided by the previous research of the originators and others which had indicated that it was possible to interpret the expression of a gene in terms of a series of biochemical reactions. (See reviews by Haldane, 1937; Wright, 1941; Beadle, 1945.) It has been suggested that the function of the gene might be ascribed to a specific reaction, that is, the mutant condition is manifested as the result of an alteration at one point in a given series of metabolic steps.

Prior to the work with Neurospora the investigations were limited to the study of characters which frequently were expressed through a series of reactions, having no known

biochemical precedence. By reversing the procedure Beadle and Tatum attempted to give a genetic basis to known physiological processes. For this purpose they utilized a plant whose synthetic capacities are such that it can be cultured on a simple medium under standard laboratory conditions and possesses a reproductive cycle compatible with genetic studies. The organism chosen was the heterothallic ascomycetous fungus, Neurospora, previously studied by Dodge (1927), Lindegren (1942) and others.

After the original, or parent, strains had been cultured on a simple media the cultures were subjected to the agents--X-ray, ultra-violet, neutrons (Beadle and Tatum, 1945), mustard gas (Horowitz, et al., 1946)--known to produce mutations. The treated material was then crossed to a strain of the opposite mating type, and the resulting ascospores were isolated on a "complete" medium. Those isolates which grew on the "complete" medium were transferred to the original minimal mixture. When no growth occurred on the latter, it was assumed that a change had been produced in the original stock so that the resultant progeny now lacked the ability to synthesize some component, or components, present in the "complete" combination. By systematic tests of the ingredients of the "complete" medium the nature of the substance required was ascertained. In this fashion mutant strains were detected which require vitamins, amino acids, purines, etc., in addition to the basal constituents on which the parental

strain grew (Beadle and Tatum, 1945).

Each mutant stock is designated by a number corresponding to the spore from which it was derived. That is, stock number "1" would represent the first spore isolated from a cross involving the treated material; "2", the second, etc. Although such a designation carries no implication as to the nature of the mutant, it remains the only practical nomenclature until a complete study of the mutant characteristics has been carried out. On a more general basis mutants are further named by adding the suffix "less" to the substance required, in other words, "argininеless" mutants require arginine; "cholineless", choline, etc.

Thus far fifteen "leucineless" mutant strains have been found. Through the kindness of Dr. Beadle these were made available to the author. A description of eight of these mutants together with an evaluation of their genetic and biochemical relationships constitutes the subject of this thesis.

A study of leucineless mutants presents an approach to several different problems. First, it would be of interest to know in what respect the gene mutation has affected the metabolism of leucine and other substances with reference to the "normal" metabolism, as exemplified by the parental stock. If the basic tenet that a gene mediates a single chemical reaction is applicable here, it should be possible to assign to each mutant strain a specific reaction. The method

frequently employed here consists in attempting to substitute for the required substances compounds which may enter into the metabolism of leucine. The choice of such substitions is guided by knowledge of the metabolism of leucine in other organisms or by the accumulated data with reference to other Neurospora mutants. Other techniques, such as, the application of specific inhibitory substances and tests for accumulated metabolic products, have also been employed.

Correlated with the establishment of the metabolic role of the mutated gene is the required genetic verification that but a single gene is responsible for these effects. This proof is derived from an analysis of the progeny from matings involving the strain and forms the second proposition to be investigated.

A third problem arising in this research was stimulated by the appearance of variations arising in the strains employed. These changes took on the aspect of reversions to the parental condition resulting in the loss of the leucine requirement.

METHODS

To measure the growth requirements of the Neurospora biochemical mutants one has several methods at his disposal. A qualitative analysis can be made by inoculations of test tubes containing media in which growth factors are incorporated. These may be scored for the presence or absence of growth after a designated time interval. In some cases the same sort of method can be employed using conidia in "hanging drop" cultures and using the presence or absence of the germination of conidiospores as an index of the growth stimulation by various types of media. The measurement by germination can be placed on a somewhat quantitative basis by determining the lowest concentration at which germ tubes are produced. As has been pointed out (Regnery, 1944; Ryan, 1946) this method combines several desirable features: (a) the tests may be made with small quantities of substances, (b) a complex medium is unnecessary since germination occurs in distilled water, and (c) the duration of the experiment is shortened, facilitating the testing of unstable compounds. The method has not found a wide application since some Neurospora mutants although possessing an exogenous growth requirement will germinate in the absence of the required substance.

A comparable but even more restricted response can be demonstrated in production of germ tubes by activated ascospores. The chief objection to such a method lies in the

potential genetic variability arising following the cross used to produce the spores.

A growth response may be measured either as a function of the rate of growth or of the amount of growth produced. Growth rates are in many cases best observed by the progression of the mycelium over an agar surface in tubes or plates. The tube method developed by Ryan, Beadle and Tatum (1943) is especially advantageous in cases where the rate of growth is not constant but changes in the course of the experiment since it permits several readings with a single treatment. As emphasized by the originators the medium which lies beyond the mycelial frontier should show no changes as the result of the mold's metabolism. Perhaps the chief disadvantages of this method lie in the number of factors (environmental and genetic) which can cause variations in the rate of growth and in the lack of discrimination between the linear progression of a few isolated hyphae and that of a solid mycelial mat and, hence, in effect the tube rate method measures the growth rate in one plane only.

The last of these objections may be obviated by recording the rate of growth in terms of the dry weight of the mycelium produced at various time intervals. The advantages of the tube rate method are the disadvantages found with liquid cultures, namely: (a) changes in the media become of some importance as the mold either depletes or enriches the media with various of its metabolites and (b) measurement of dry weights of

necessity entails the cessation of growth, therefore one must deduce the growth rate by employing many flasks with identical contents over an extended period of time. The usual technique consists of designating an arbitrary time limit to the experiment and recording the dry weight of the mycelium which has accumulated up to this point. Such a procedure has its limitations since no account is taken of the particular phase or rate of growth at which the experiment was terminated.

In some cases it is possible to measure the maximum or "total" amount of growth supported by the medium. This again requires the determination of weights at several time intervals up to a point after which no increase is observed. In this way it is possible to compare the activity of compounds not in terms of the rate of their utilization but rather on the basis of the relative over all utilization. Since this method likewise will measure growth rates it combines several advantageous features and gives a deeper insight as to the type of growth response. It becomes somewhat impractical in experiments where the number of variables is great because it frequently entails twenty or more determinations with each treatment.

These methods have been used in the study of the strains of Neurospora which respond to leucine. The method of choice frequently varied with the mutant and with the type of treatment. To make a basic comparison among those mutants studied, growth curves were made on the basis of dry weight

obtained in liquid cultures. The details of the method are briefly summarized in the following:

The modified Fries biotin mixture (Beadle, 1945) was made up to twice the concentration at which it would be employed. Sucrose and the substances to be tested were similarly made up to twice the desired final concentration in distilled water. Equal portions of each solution were combined and from the resultant solution 10 ml. were distributed into 125 ml. Erlenmeyer flasks. The flasks were plugged with cotton and autoclaved for fifteen minutes at fifteen pounds pressure. After cooling the flasks were inoculated with a drop from a suspension of conidia of the strain to be tested. Stocks of the leucine mutants from which the conidia were derived, unless otherwise designated, were maintained on slants containing the "minimal mixture"--2% Bacto-agar, 2% sucrose and .1 mg.% l(-)-leucine. The conidia were suspended in distilled water and although it is impossible to achieve a standard dilution, the criteria most frequently employed was the lack of any discernible opacity. This constitutes a "dilute" conidial suspension by comparison with most other Neurospora workers.

After inoculation the cultures were maintained at 25° C. until the mycelium was harvested. In all of the later experiments routine shaking by hand to reduce sporulation was avoided since it was deemed impossible to rule out an appreciable variability with this technique. The mycelial mass

was removed and after the excess moisture was expressed, dried at 100° C. to a constant weight. The mycelial weights were measured on an analytical balance to the nearest tenth of a milligram.

All chemicals used were designated as "chemically pure", unless indicated otherwise, and all glassware used was cleaned with a sulphuric acid-chromate solution.

PHYSIOLOGY

Introduction

The growth response to leucine of these mutants may be classified into four general types (Figure 1). In the first category one finds an absolute dependence on leucine. In the absence of this amino acid no growth takes place and the maximum mycelial dry weight is directly proportional to the total amount of leucine available. The second type is a modification of the above and under some conditions will show the same relationships. It differs in that following leucine depletion secondary growth may occur giving an additional increase in the mycelial weight.

The third type of response may be classified as a "partial" requirement. In the absence of leucine growth may take place and be sustained at a rate comparable to that of the "wild type". However, there may be a prolonged lag phase, the duration of which is greatly shortened by supplementary leucine. The last type also indicates a "partial" requirement, but the growth rate on "minimal" medium is slower than that obtained on media containing leucine or of the "wild type" controls.

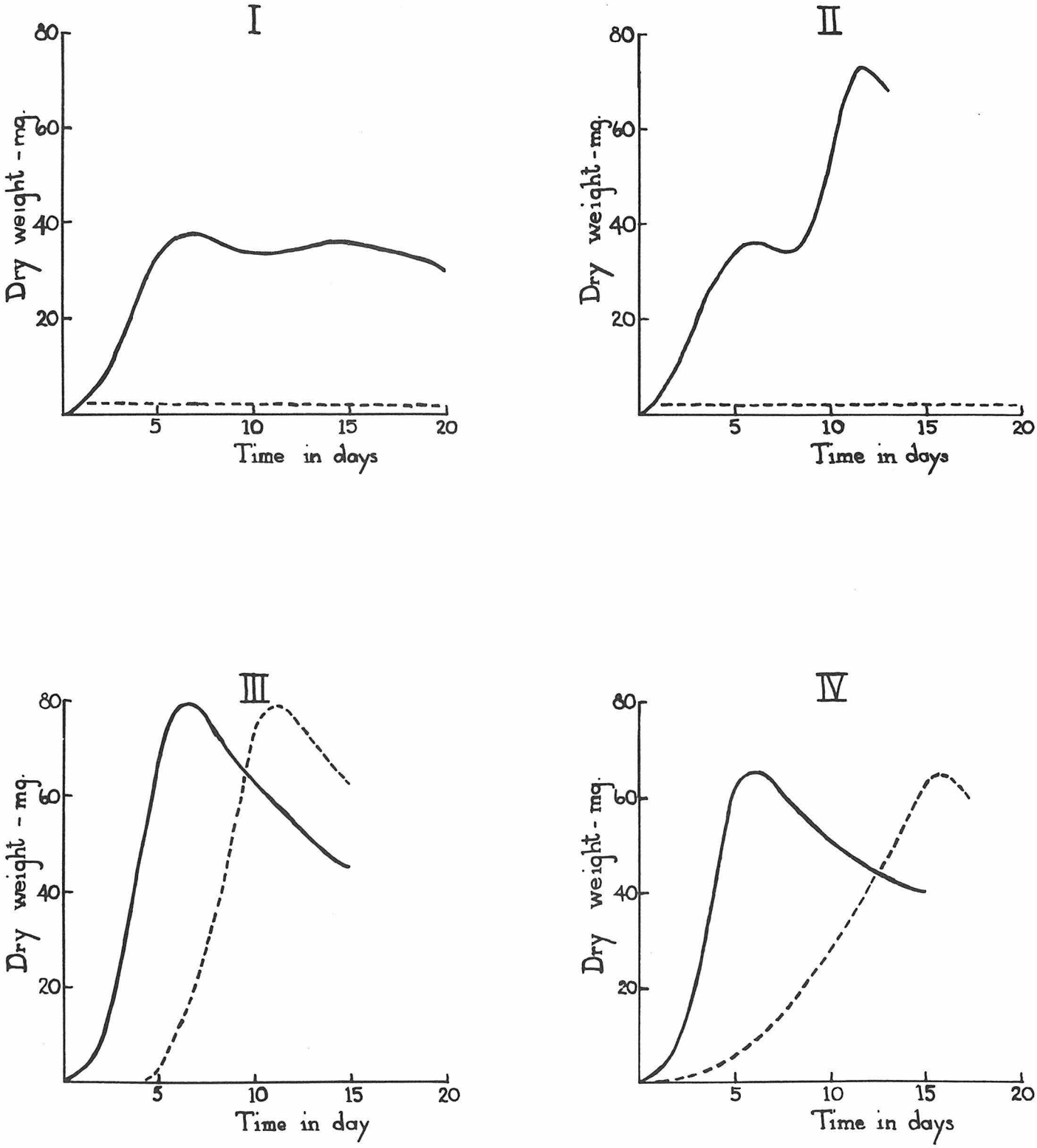
It should be emphasized that these growth responses are obtained under similar environmental conditions. Variation of one factor, such as, temperature, pH or amount of sucrose, can shift a particular strain from one category to another. Moreover, these groupings are not necessarily coincidental with

FIGURE 1

TYPES OF GROWTH RESPONSES
DISPLAYED BY THE LEUCINELESS MUTANTS

The solid line represents mg. of dry weight with 1 mg. l(-)-leucine and 200 mg. sucrose/10 ml. of culture medium; broken line, growth on 200 mg. sucrose/10 ml. of culture medium.

FIGURE 1


I
II
III
IV
Dry weight - mg.
80
60
40
20
5
10
15
20
Time in days
Time in day

a genetic classification. It is simply an arbitrary standard, or yardstick, by which one can compare and evaluate the growth response and physiology of the different strains or mutants.

GROUP I

33757

Mutant strain 33757 was derived from material treated with ultra-violet radiation. It has been the most extensively studied strain of the leucineless group and was chosen as the most suitable for microbiological assays for leucine (Regnery, 1944; Ryan and Brand, 1944). Subcultures of the original and of the genetically derived strains of 33757 have been maintained for the last five years on agar slants containing minimal medium fortified with leucine, and under these conditions the stocks have retained the specific characteristics of the original culture. Of the amino acids tested only leucine supports the growth of the mutant. A negative response was noted with each of the following: dl-alanine, l(+)-arginine, l(-)-asparagine, l(-)-cystine, l(+)-glutamic acid, glycine, l(-)-histidine, l(-)-hydroxyproline, dl-isoleucine, l(+)-lysine, l(-)-methionine, dl-norvaline, dl-norleucine, dl-phenylalanine, l(-)-proline, dl-serine, dl-threonine, l(-)-tryptophane, l(-)-tyrosine, and dl-valine.

Germination--The lack of growth response in the absence of leucine is further reflected in the inability of the conidia to germinate in leucine-deficient media. This response was observed in hanging drop cultures by making conidial suspensions in various concentrations of leucine. Where the concentration of l(-)-leucine was less than .5 gamma/ml. none of the conidia appeared to produce hyphae. With the higher concentration at 25° C. signs of germination usually appeared within four to five hours, the time being dependent on the concentration, on the age of the culture from which the spores were derived, the size of the spores and the degree of dessication. The response was noted in distilled water and leucine, hence, there was apparently a sufficient storage of material within the conidia to support, at least, the begining of hyphal growth.

Heat activated ascospores carrying the mutant gene 33757 likewise fail to produce germ tubes in the absence of leucine. Addition of leucine to tubes containing such spores resulted in the production of hyphae, the amount of mycelia depending upon the amount of leucine added.

Leucine Growth Response--The quantitative dependence of mycelial growth on available leucine is illustrated in Figure 2. The strain used in this case was a double mutant, 33757, 4637A (albino), one which has been subcultured for four years on leucine enriched medium. In all cases the medium contained in addition to the leucine, 200 mg. of sucrose and

FIGURE 2

DRY WEIGHTS OF MYCELIA PRODUCED BY 33757
FOR VARYING TIMES ON VARYING AM'TS
OF LEUCINE

Cultures were grown at 25° C. in 10 ml. of 2% sucrose. The amounts of leucine are indicated for each curve. Each point represents the average calculated from three determinations.

FIGURE 3

RELATION OF MAXIMUM MYCELIAL DRY WEIGHT
PRODUCTION BY STRAIN 33757 TO
l(-)-LEUCINE CONCENTRATIONS

The cultural conditions are the same as those indicated for Figure 2.

FIGURE 2

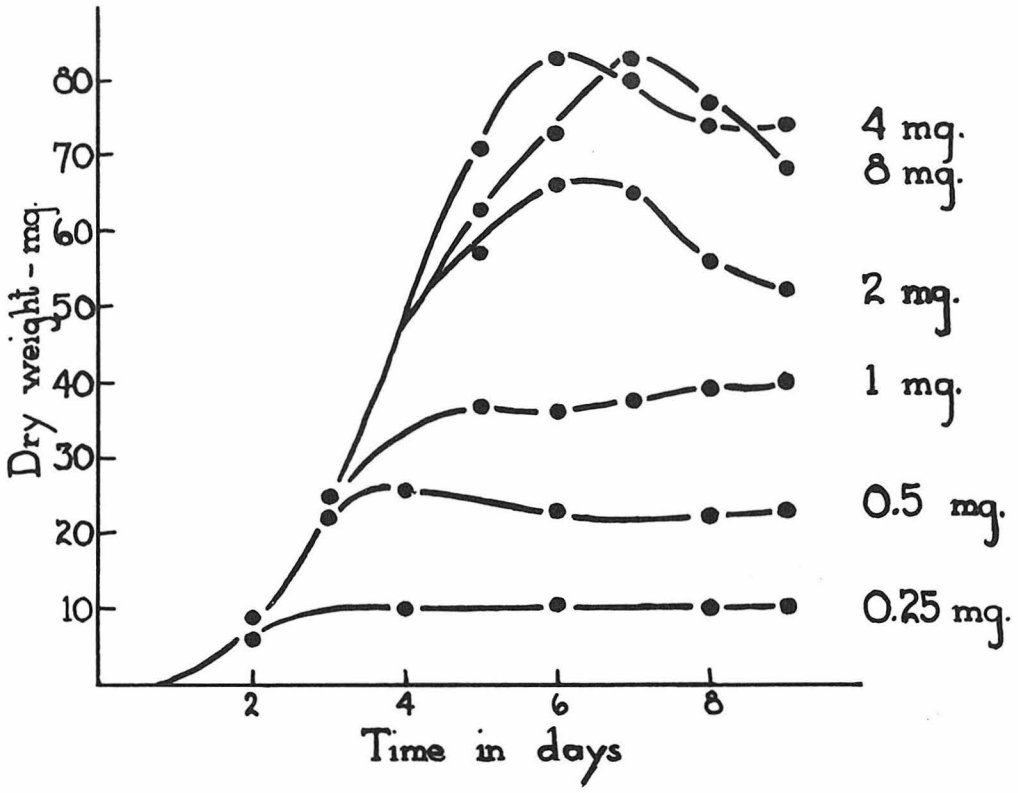
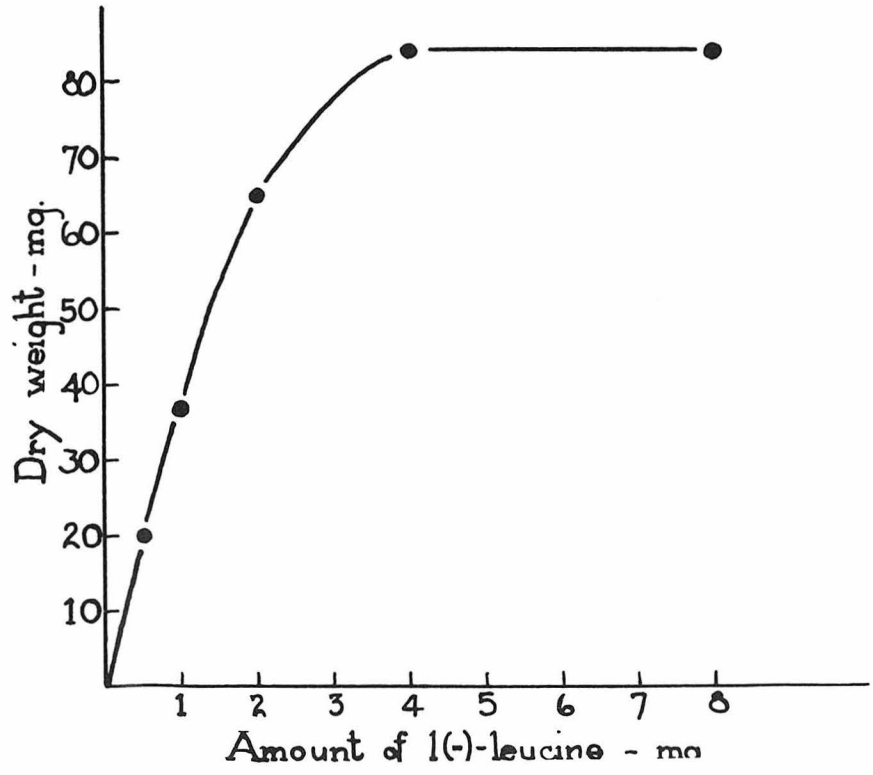


FIGURE 3



10 ml. of the standard Fries biotin combination. None of the flasks were shaken. A plot of the maximum weights obtained in a representative experiment is shown in Figure 3. That leucine is the principal limiting factor in cases where less than 2 mg. of leucine are used can be demonstrated in two ways. The depleted medium may be sterilized after the mycelium has been removed and reinoculated with either wild type or 33757. In the first case growth will occur; in the second, the further addition of leucine is necessary to support growth.

It is improbable that all of the leucine initially present in the medium is utilized for growth. In view of the experimental evidence it is more likely that the utilization is accompanied by a destruction of leucine. This was demonstrated by culturing the organism on media which is deficient in the amount of sucrose necessary to bring about the most efficient leucine utilization. After five days the sucrose deficit was restored, and it will be noted that the increase in dry weight was considerably less than that expected if there had been no leucine destruction or if the leucine were "stored" in some fashion (Table I). The manner in which leucine is broken down is not clear. No end products have been identified by chemical means. When excessive amounts of leucine were employed, however, an odor which strongly resembled that of isoamyl alcohol developed between the fifth and seventh days of growth.

Effect of Carbon Source--If the amount of sucrose

is increased relative to the leucine initially present, it should be possible to attain conditions where there exists an excess of the carbon source and thereby to limit the growth with leucine alone. However, it was found that the amount of sucrose will markedly influence the dry weight values even where there is an "excess" used. Tests of the medium from a

TABLE I

Initial Amt. Sucrose (mg.)	l(-)-Leucine (mg.)	Dry Weight	
		5 days (mg.)	10 days (mg.)
20	1	8.7	5.7
		8.4	5.6
		8.8	5.7
20 (180 added after 5 days)	1		16.7
			16.9
			17.6
200	1		40.4
			39.2
			40.0

seven day culture of 33757 containing 200 mg. sucrose and 1 mg. l(-)-leucine/10 ml. showed that at this time the filtrate will allow at least 30 mg. of mycelial dry weight of the wild type. The amount of 33757 mycelium removed at seven days was 40.5 mg. giving a minimum total of 70 mg. of dry weight for 200 mg. of sucrose. This is in fair agreement with the observation that the maximum mycelial dry weight of the leucineless mutants is equal to roughly 45% of the initial sucrose concentration.

The increase in weight with increased sucrose is not a function of the concentration of sucrose since the same dry weight values are obtained with 1 mg. of l(-)-leucine in 10 ml. of 2% sucrose as with 1 mg. l(-)-leucine in 20 ml. of 1% sucrose.

TABLE II

Volume of Medium (ml.)	Initial Amt. Sucrose (mg.)	l(-)-Leucine (mg.)	Dry Weight	
			5 days (mg.)	10 days (mg.)
10	200	1	32.4	33.3
			32.4	33.6
			34.3	32.7
20	400	1		40.5
				40.2
				40.8
10	200 10 ml. 2% sucrose added after 5 days	1		42.4
				43.9
				43.4
10	200 10 ml. Fries media added after 5 days	1		30.6
				30.6
				30.4

Furthermore, the "excess" sucrose was added after the active growth period and still the increase in mycelial weight was apparent (Table II).

Some care must be taken in the analysis of these increased weights as the appearance of "reversions" becomes more likely as the amount of sucrose is increased relative

to the dry weights. This topic will be discussed under a separate heading.

A similar increase was obtained with a variety of sugars (Figure 4), the only clear exception being the pentose, xylose. These values represent dry weights, where 1 mg. of l(-)-leucine was used as a common factor. The same relationships hold when lesser amounts of leucine are used and the increases are of the same relative order of magnitude as with 1 mg. Thus, it becomes apparent that the addition of different amounts of leucine to a common substrate should yield weights which deviate from a direct dry weight proportionality.

TABLE III

l(-)-Leucine (mg.)	Dextrose (mg.)	Acid Added (100 mg.)	Dry Weight 7 days
1	100	-----	26.7
1	100	Citric	30.6
1	100	Malic	33.0
1	100	Succinic	35.0

Other substances tested for stimulatory action included citrate, sucinate and malate. Although these compounds did not in themselves support measurable amounts of growth, they did in the presence of glucose cause an increase in dry weight (Table III). It should be pointed out that in such a case citric acid should not be used in buffering mixtures if pH effects are to be studied.

FIGURE 4

RELATION OF DRY WEIGHT PRODUCTION
BY STRAIN 33757 TO VARIOUS CARBON SOURCES

The cultures contained 1 mg. l(-)-leucine/10 ml. of medium and were grown in 125 ml. flasks for 7 days at 25° C. Each point represents an average of three determinations.

FIGURE 5

RELATION OF DRY WEIGHT PRODUCTION
BY STRAIN 33757 TO ISOVALERIC ACID
ADDED TO THE MEDIUM

Additions of isovaleric acid are expressed in ml./10 ml. of culture medium in 50 ml. flasks.

FIGURE 4

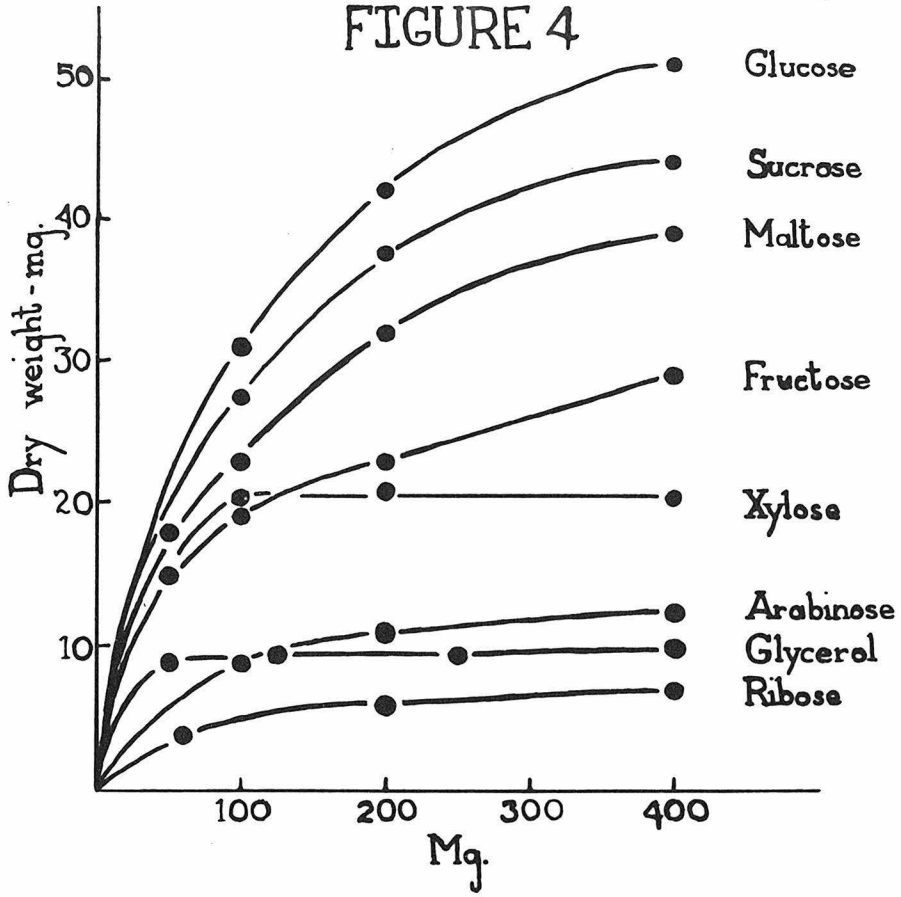
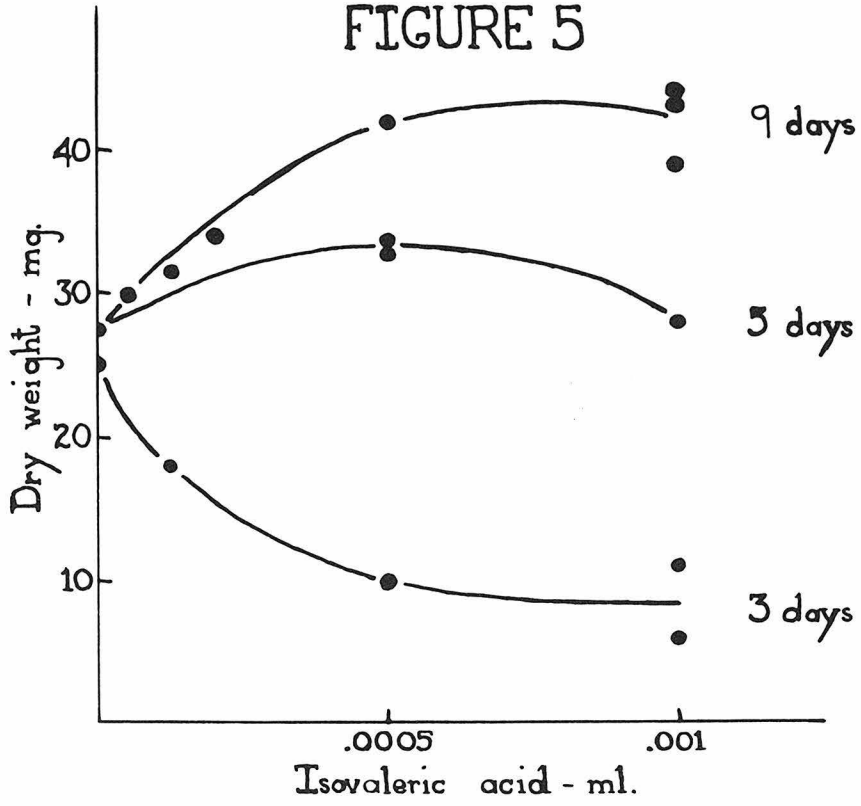


FIGURE 5



Another variable with respect to the carbon source was found with different periods of autoclave sterilization. With filter-sterilized media the growth was found to be less than that on media which were autoclaved for 15 minutes at 15 pounds pressure; when autoclaved for one hour a further increase was obtained. The increase was slightly more striking if the sucrose was autoclaved in distilled water and later added to the leucine containing basal medium. Glucose behaved similarly whereas fructose showed little change upon such treatment (Table IV).

TABLE IV

AVERAGE DRY WEIGHTS
OBTAINED AFTER 7 DAYS GROWTH

Sugar	Duration of Autoclave Sterilization		
	15 minutes (mg.)	1 hour (mg.)	1 hour Sugar separately (mg.)
Sucrose	45.2	46.8	49.4
Dextrose	44.7	42.1	51.2
Fructose	25.6	23.2	25.6

Rate of Growth--As the mycelial weight is dependent upon available leucine, so the rate of growth on an agar medium depends upon the concentration of the amino acid. Table V shows the growth rates of 33757, 4637A on different concentrations of leucine. The values with the lower concentrations of leucine were frequently rather variable from day to day as the growth pattern was somewhat erratic. The rate

of growth can be measured in liquid cultures, and it was found that continuous agitation, or rotation, of the flasks, while shortening the time at which the cultures showed maximum growth, also caused a decrease (28%) in the dry weight obtained when compared with the stationary controls. This effect was reflected in those instances where the flasks were shaken twice daily, although the decrease in dry weight was not as striking.

TABLE V

Concentration l(-)-Leucine (gamma/ml.)	Rate of Growth (mm./hr.)
100.	3.3
50.	3.4
25.	3.2
12.5	2.6
6.25	2.3
3.12	.5

2% sucrose, 2% agar.

A decrease (48%) in "final" dry weight was, likewise, obtained when the temperature at which the mutant was cultured was raised to 35° C. Here again the rate increase is apparent. It is obvious then that the growth conditions must be rigidly defined to obtain reproducible results.

Response to dl-Leucine, Alpha Ketoisocaproic Acid and Leucic Acid--The above has referred exclusively to effects obtained on l(-)-leucine, the naturally occurring isomer. With

synthetic leucine, which contains both d and l forms, the results were essentially similar with some exceptions. Notably the growth rate as measured by the tube method was lower in the case of dl-leucine than with a similar concentration of the l form. However, this difference disappeared when concentrations of greater than 25 gamma/ml. were used. In this case the two compounds possessed similar activity.

TABLE VI

Time of Incubation (days)	Volume of Medium (ml.)	Dry Weight	
		dl-Leucine (mg.)	l(-)-Leucine (mg.)
3	10	24.4	25.1
	20	19.1	
	30	17.4	
7	10	29.0	28.1
	20	24.0	27.6
	30	19.2	27.2
9	10	30.8	29.2
	20	25.4	
	30	23.3	

The same comparison is found in flasks where an increase in the dilution of the synthetic compound decreased greatly its initial growth promoting activity (Table VI). It was shown that filter-sterilized d(+)-leucine was inactive when present as the leucine source in media. If autoclave sterilization was utilized, some growth occurred suggesting that racemization

had taken place. The activity of the racemic form is best explained by assuming that in the presence of growing mycelia utilization of d(+)-leucine is possible. This is supported by our knowledge (Horowitz, 1944) that Neurospora can elaborate a d-amino acid oxidase, which may oxidize the unnatural isomer to the keto acid analogue and that this in turn may be reformed into l(-)-leucine. The development of such a system may be considered analogous to the "adaptive" enzymes found in Neurospora and other organisms.

Enzymatically prepared alpha-ketoisocaproic acid was found to be fully capable of replacing the leucine requirement of 33757. Quantitative data were somewhat unreliable in this case since there was some question about the purity of the preparation. A synthetic preparation of the alpha-hydroxy acid analogue, kindly furnished by Dr. D. Bonner, was as active as l(-)-leucine on a comparable molar basis. As in the case of d(+)-leucine, the ability to use dl-leucic acid was adaptive in nature since with small inocula no growth occurred on the compound alone. Either a mycelial inoculum or the addition of small amounts of l(-)-leucine (.02 mg./10 ml.) was sufficient to initiate a growth response. Variations in pH over the range of 3.9 to 8.0 and in temperature from 20° to 35° C. did not result in the initiation of growth on the hydroxy acid.

Effect of Other Substances-- On the supposition that other compounds related to leucine, or to the metabolism of

leucine, might satisfy the growth requirements of 33757, the following substances were tested individually and in the presence of leucine: acetic, propionic, n-butyric, isobutyric, n-valeric, isovaleric, n-caproic and isocaproic acids, acetone, isobutyraldehyde, isovaleraldehyde, isoamyl alcohol and isoamylamine. In the absence of l(-)-leucine none of these showed activity. When added to media containing leucine, however, isovaleric acid, isovaleraldehyde and to a lesser extent isoamyl alcohol markedly increased the yield of mycelium. For example, 0.001 ml. of isovaleric acid or isovaleraldehyde and 1 mg. of l(-)-leucine in 10 ml. of media increased the nine day yield from 28 mg. to 42.5 mg. (Figure 5). A similar increase was produced by the addition of 0.5 ml. of isoamyl alcohol. Higher concentrations of these compounds were inhibitory. All attempts to obtain growth on isovaleric acid or isovaleraldehyde alone by transferring from a medium containing l(-)-leucine were negative. In all cases cultures yielding high dry weights were checked and failed to grow on leucine-free media, showing that neither reversion nor contamination had occurred.

This additive effect of isovaleric acid or isovaleraldehyde is abolished if the cultures are grown on a shaker. Furthermore, "deep" cultures gave a more pronounced increase while cultures of the same volume and constituents in larger flasks yielded lower dry weights. Since the growing conditions seem to effect the isovaleric acid increase, the compound

was added at ten days, or after the actively growing period was over. No increase was noted.

It is known that in the catabolism of leucine in other froms isovaleric acid can be formed either by the decarboxylation of alpha ketoisocaproic acid (Embden, 1912) or from the oxidation of isoamylamine (Loeffler, 1916), which in turn is derived by the decarboxylation of leucine. The possibility that the last reaction may be reversible is suggested by Lyman, et al. (1947), who have shown in Lactobacillus arabinosus that carboxylation may occur with arginine, phenylalanine and tyrosine. If Neurospora can carboxylate isovaleraldehyde, one might expect to find such a reaction occurring most readily where a high carbon dioxide tension exists. This may satisfactorily explain the differential effects of "deep", or stationary, cultures and the shallow, or shaken, cultures. All attempts to obtain growth on isovaleraldehyde with increased carbon dioxide tension yielded negative results.

Another possible explanation of the isovaleraldehyde effect involves the unproved assumption that in Neurospora leucine is degraded through isovaleraldehyde and the corresponding acid. If this were true, it is possible that increased amounts of these compounds would exert a "sparing" action by altering the equilibrium of the degradative reaction. The addition of isovaleraldehyde after the actively growing phase would be expected to have little effect since by this time most of the free leucine initially present would have been metabolized. Also, the percentage increase with added isovaleric acid is proportional to the amount of l(-)-leucine

initially present.

Inhibitory Compounds--It was noted above that the growth of 33757 was not supported by any of the other amino acids tested. The same compounds were added to leucine cultures to ascertain if any might partially replace the leucine requirement (cf. Bonner, et al., 1943). In concentrations of 1 mg./10 ml. of medium with 1 mg. of l(-)-leucine none showed a striking increase or decrease in dry weight. The rate of growth was markedly influenced in some cases, most notably by dl-phenylalanine, l(-)-tyrosine and dl-norleucine. In liquid medium where the concentration was twenty times that of the leucine present complete inhibition by dl-norleucine was obtained. The inhibitory effects of these compounds were more strikingly demonstrated in solid media where there is little chance of a decrease in the concentration of the inhibitors.

Growth on Peptides and Intact Proteins--In 1901 F.A.F.C. Went demonstrated that Neurospora was able to elaborate proteolytic enyzmes if grown on certain types of substrata. 33757 was able to satisfy its leucine requirement when some unhydrolyzed proteins and peptides were used as a source of the amino acid. Thus, dl-leucyl glycine and glycyl-l-leucine were utilized by this strain, although in each case growth is initially slower than on l(-)-leucine.

It is apparent in these two cases that the mutant was able to utilize the leucine, regardless of whether the amino or carboxyl group is involved in the peptide linkage.

The final weights attained on glycyl-l-leucine were equal to those induced by an equimolar quantity of leucine, while those attained with dl-leucylglycine were lower than would be expected on the basis of complete hydrolysis and utilization. Leucyldiglycine was apparently not hydrolyzed, since a mixture with leucine gave values no greater than the leucine controls. Tested in the same fashion, glycylleucylglycine showed slight activity. Since the production of peptidases frequently is increased by growth on unhydrolyzed proteins, attempts were made to obtain growth on the three inactive peptides by making use of mycelial transfers from cultures growing on gelatin, casein, egg albumin or glycyl-l-leucine. In all cases these attempts were unsuccessful.

The leucineless mutant can hydrolyze proteins and thereby satisfy its leucine requirement if mycelial inocula are used or if growth is started by adding a small amount of free leucine. Casein and zein produced abundant growth in five days. Hemoglobin and gliadin were likewise rather readily broken down; gelatin and egg albumin were more slowly utilized. Table VII summarizes preliminary data on the leucine content of casein. The results obtained with the unhydrolyzed protein, an acid hydrolysate, and a tryptic digest are in reasonable agreement.

One difficulty in obtaining consistent quantitative data with respect to the leucine content of unhydrolyzed protein lies in the inability to disentangle the mycelial mass from residual unhydrolyzed protein. Growth tubes cannot

TABLE VII

Treatm't of Casein	Casein (mg.)	l(-)-Leucine (mg.)	Incubation Period (days)	Dry Weight (mg.)	Calculated Leucine (mg.)	Leucine Content (%)	Recovery of added Leucine (%)
HCl-hydrolyzed	10	0	8	31.1	1.1	11	
				32.8	1.2	12	
	5	0	8	19.8	0.65	13	
				20.7	0.70	13	
	5	0.5	8	29.7	1.0		91
				30.8	1.1		
	0	0.5	8	15.9			
				16.1			
Tryptic-hydrolyzed	10	0	5	31.6	1.1	11	
			7	31.5	1.1		
	5	0.5	5	30.3	1.05		100
			7	30.2	1.05		
	0	1.0	5	29.8			
			7	28.1			
Unhydrolyzed	10	0	8	29.7	1.05	10.5	
				30.5	1.05	10.5	
				31.1	1.1	11.0	
			10	29.6	1.05	10.5	

be used since the rate of growth will not only depend upon the potentially available leucine but also on the rate of hydrolysis. In no case was an attempt made to obtain enzyme activity in the filtrate or cell free extracts.

Recently Hodson and Krueger (1947) have utilized the ability of this strain to hydrolyze intact proteins as a means of bioassay. Their results are in good agreement with the author's. They, too, point out the inapplicability of this method where fibrous compounds are to be assayed.

46409

The second mutant of Group I is 46409, also derived from a wild type by ultra-violet treatment. In most respects it was similar to 33757 in regard to its biochemical responses. It differed chiefly in having a slower growth rate (1.2 mm./hr. on 100 gamma l(-)-leucine/ml.) and in showing proportionally greater increase with supplementary isovaleric acid or isovaleraldehyde and, also, a more pronounced inhibition with added dl-phenylalanine or dl-norleucine. As in the case of 33757, 46409 showed a sucrose stimulation. Most striking was

TABLE VIII

l(-)-Leucine (mg.)	Sucrose (mg.)		Dry Weight 10 days (mg.)
1	500	Isovaleraldehyde .0005 ml.	50.3
1	200	Isovaleric Acid .0005 ml.	39.1
1	500	-----	40.4
1	200	-----	31.5

the growth on media containing 10% sucrose and 1 mg. l(-)-leucine/10 ml. After attaining a weight of 40.8 mg. in eight days dry weight increases were noted on each successive day until weights of 174.8 mg. were reached in 30 days. Tests of the cultures revealed no evidence of reversion. 46409 can utilize calcium leucate provided there is an initial adaptation.

70311

Strain 70311 is the third leucineless mutant of Group I. It resembles 33757 in its ability to utilize the hydroxy analogue of leucine and to show an increase in weight with isovaleric acid, isovaleraldehyde and "excess" sucrose. 70311 grew at a rate of 2.5 mm./hr. on 100 gamma l(-)-leucine/ml. However the rate did not remain constant but gradually decreased to 2.3 mm./hr. The negative acceleration was more noticeable in tubes containing 25 gamma l(-)-leucine/ml. where the initial rate was 2.1 mm./hr. and decreased to a value of 1.5 mm./hr. after five days. It was inhibited by dl-phenylalanine and dl-norleucine, again showing a parallel deceleration with each substance. Since this mutant has exhibited no tendency to revert to a wild type condition it was chosen to illustrate the effect of isovaleric acid over an extended period of time, Figure 8. When 70311 was cultured

FIGURE 6

DRY WEIGHT OF MYCELIA PRODUCED BY
STRAIN 8839 GROWN FOR VARYING TIMES.

Each flask contained 1 mg. of l(-)-leucine and 200 mg. sucrose/10 ml. of culture medium. The amount of growth of 33757 on the filtrate of 8839 cultures taken off at different times is indicated by a broken line.

FIGURE 7

GROWTH RATES OF 8839 ON VARIED
CONCENTRATIONS OF
l(-)-LEUCINE, dl-LEUCIC ACID AND dl-LEUCINE

Inoculated by mycelial transfer; temperature, 25° C.; sucrose and agar concentrations, 2%.

FIGURE 6

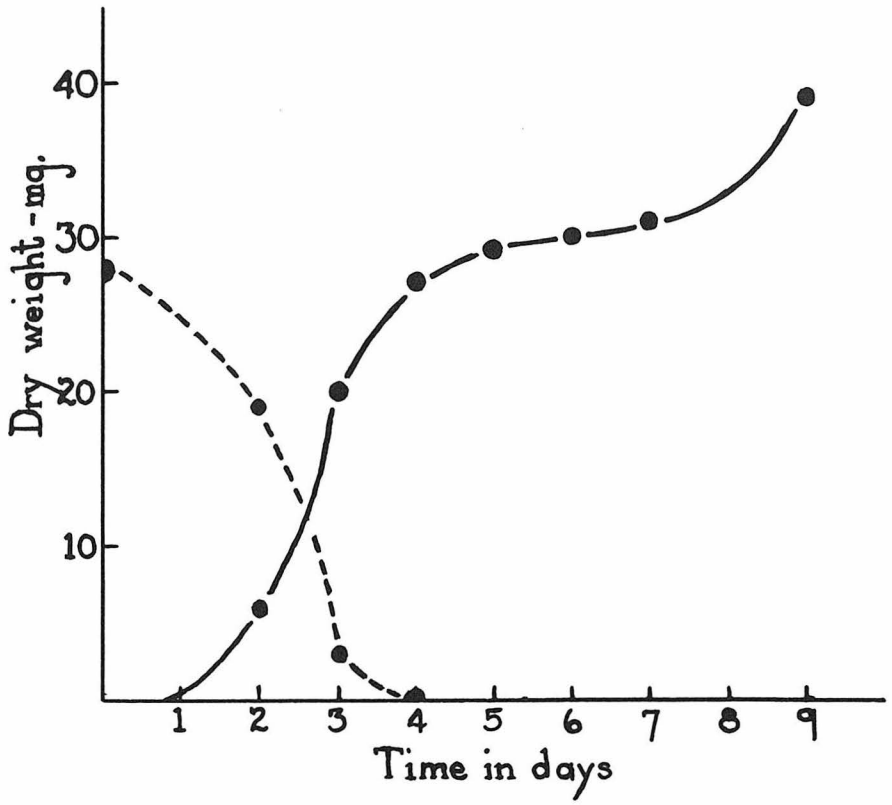


FIGURE 7

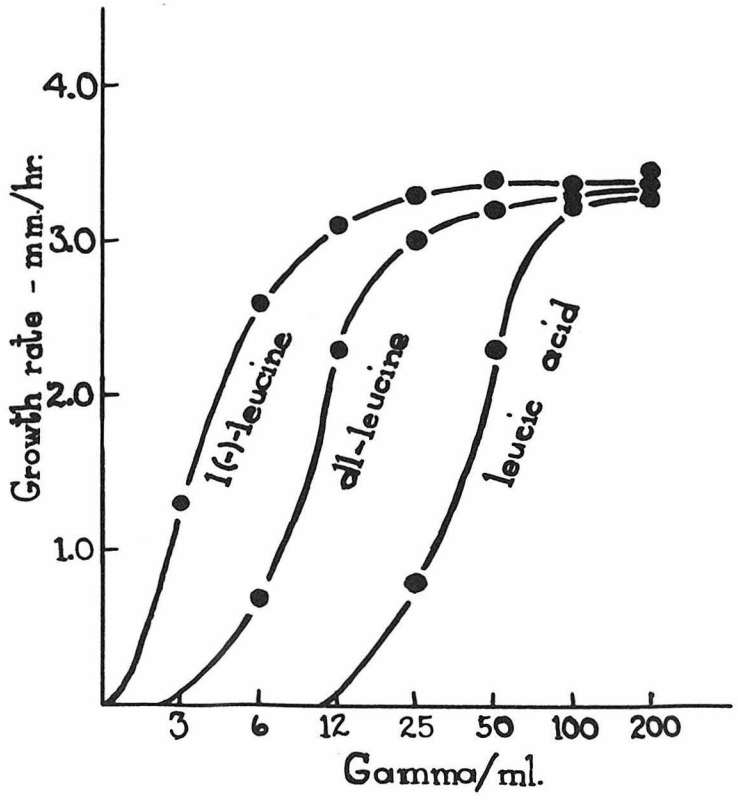


FIGURE 8

GROWTH CURVE OF STRAIN 70311 GROWN ON
1(-)-LEUCINE SUPPLEMENTED WITH
ISOVALERIC ACID

Solid line indicates dry weights obtained on 1 mg. 1(-)-leucine and 200 mg. sucrose/10 ml. culture medium; broken line indicates dry weights on the same medium to which .0005 ml. of isovaleric acid was added.

FIGURE 9

GROWTH CURVE OF STRAIN 47313 ON
1(-)-LEUCINE AND MINIMAL MEDIUM

Solid line indicates dry weights obtained on 1 mg. 1(-)-leucine and 200 mg. sucrose/10 ml. culture medium; broken line indicates growth on 10 ml. minimal media and 200 mg. sucrose.

FIGURE 8

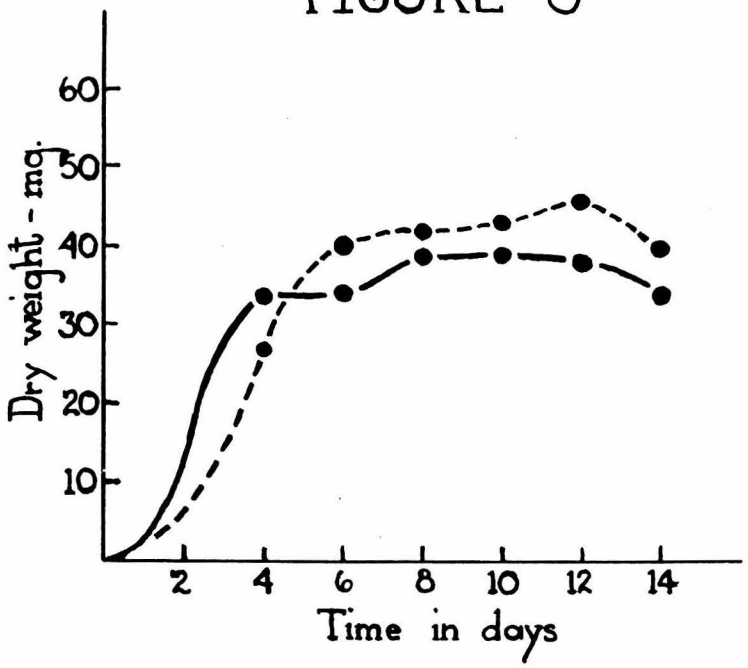
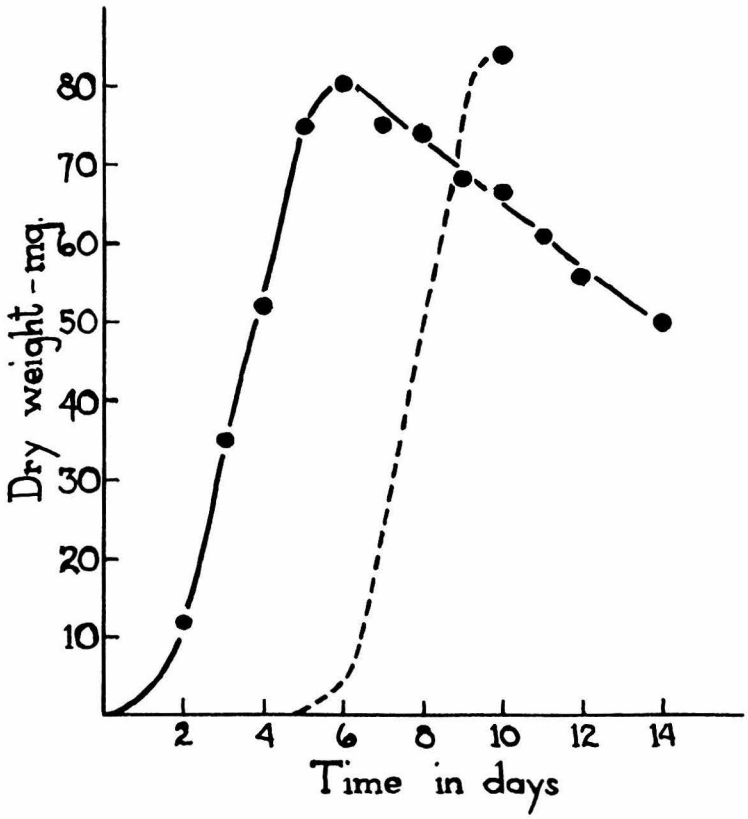


FIGURE 9



for 30 days on 1 gm. sucrose and 1 mg. of l(-)-leucine, no dry weight increases were observed after the ninth day. In the same type of medium 33757 reverted to wild type in 100% of the cultures. Thus far 70311 stands out as being the most stable of the leucineless mutants.

GROUP II

8839

Group II is represented by the mutant 8839, a strain arising from X-rayed perithecia. Although similar to 33757 in many respects 8839 differs in that following the depletion of leucine from the media a secondary overgrowth of the mycelium occurs (Figure 6). The change from the leucine-requiring strain to one which can grow in the absence of leucine appears abruptly. In liquid cultures the alteration in growth type may immediately follow the cessation of growth or may take place after a slight pause. For this reason it was not possible to evaluate responses in terms of "total growth" because dry weight values for all leucine concentrations, exclusive of the minimal controls, might depend on factors other than leucine.

A clearer representation of the "reversion" of 8839 was found in growth tube experiments. If a small

inoculum was used, a concentration curve could be constructed as illustrated in Figure 7. However, after several days in tubes, where the mycelial progression was limited to .6 mm/hr., the rate sometimes suddenly increased to 4.1 mm./hr. accompanied by strong conidiation and other characteristics ascribable to wild type strain. The same sort of change occurred in higher concentrations of leucine after the agar surface had been covered with mycelium. The reversions appeared as heavy patches at different points throughout the length of the tube, overgrowing the surface and merging to form a solid mat of heavy growth. Inocula taken from such patches or from the end of an "accelerated" culture produced cultures which grew on leucine-free medium. The reversions also occurred on minimal medium, if a large inoculum was used. It was found that the most feasible way to maintain an unreverted stock was to transfer conidia or mycelia from growth tubes containing 100 gamma leucine/ ml. of culture medium to fresh tubes of the same composition. Stocks maintained in test tubes vary greatly in their stability and may revert quite frequently. The physiology and genetics of such changes will be dealt with in the last section of this thesis.

Using the growth tube technique other amino acids were tested for synergistic or inhibitory effects (Table IX). None of these compounds would support growth of 8839 in the absence of l(-)-leucine. As can be seen from the data, with the possible exception of dl-serine, no compound increased the

TABLE IX

EFFECTS OF EQUIMOLAR CONCENTRATIONS OF SUPPLEMENTARY AMINO ACIDS ON THE GROWTH RATE OF 8839

Supplementary Amino Acid* (.0002 M.)	Growth Rate (mm./hr.)
-----	2.9
dl-Serine	3.0
l(+)-Glutamic Acid	2.9
Glycine	2.9
l(+)-Lysine	2.8
l(-)-Asparagine	2.8
l(+)-Ornithine	2.8
dl-Isoleucine	2.8
dl-Threonine	2.8
l(-)-Proline	2.7
dl-Alanine	2.7
l(-)-Histidine	2.7
l(-)-Hydroxyproline	2.7
l(+)-Arginine	2.7
l(-)-Cystine	2.7
dl-Valine	2.7
dl-Norvaline	2.7
d(+)-Isoleucine	2.7
l(-)-Methionine	2.4
l(-)-Tryptophane	2.4
l(-)-Tyrosine	1.3
dl-Phenylalanine	0.5
dl-Norleucine	0.01

* Basal concentration .0002 M. dl-Leucine.

growth rate and a few were decidedly inhibitory. l-Tyrosine, dl-phenylalanine and dl-norleucine were found to be very toxic.

Synthetic leucine and dl-leucic acid gave growth rates as indicated in Figure 7. Mycelial inocula were used with the hydroxy acid since conidial suspensions did not result in growth. Although dl-leucine and dl-leucic acid appeared to be less active (50% and 10%, respectively) than l(-)-leucine in supporting growth, the amount of dry weight obtained in cultures where reversions did not occur were similar for equimolar solutions. Keto acid prepared from hog kidney treated dl-leucine was also active. The effects of isovaleric acid could not be tested on this mutant since the growth rate depression eliminated the possibility of using growth tubes.

GROUP III

47313, 5936 and 66108

The growth response of 47313 to l(-)-leucine is represented in Figure 9. It is apparent that the requirement for leucine under these conditions cannot be considered as entirely limiting for an expected cessation of growth upon leucine depletion was not observed. Variations in initial concentrations of leucine did not result in differences in dry weights, if the growth period was extended to seven days. Rather the function of leucine in this case seemed to be one of stimulation which is expressed as a shortening of the

"lag" phase of growth. If a mycelial inoculum was used, growth occurred in media devoid of leucine. This type of response has been noted in other Neurospora mutants and has been variously described as "adaptation" or the expression of a partial or an incomplete block in the synthesis of the required substance. It would appear analogous to the response of 33757 to leucic acid or the unnatural isomer of leucine.

The need for an exogenous supplement may be brought about with a lowered pH or an increase in temperature. Minimal media buffered at a pH of 3.5 prolonged the "lag" phase of 47313 for at least seven days, unless leucine was added. The same effect was noted in cultures maintained at 35° C. Inocula from older, somewhat dessicated cultures frequently showed no signs of growth in the absence of leucine. If germinated on minimal medium, ascospores bearing the gene 47313 produced very weak growth, whereas on leucine fortified media a similar spore produced growth almost indistinguishable from that of several wild type strains.

By making use of dilute inocula the activation response of this mutant was tested on several amino acids other than leucine. Although delayed the response occurred first in l(+)-arginine and shortly thereafter in l(-)-proline solutions. Other amino acids eventually acted similarly with the exception of dl-phenylalanine and l(-)-tryptophane supplements neither of which proved to be better than the minimal medium.

Perhaps the most instructive type of response was

to a filtrate of a pyridoxineless mutant. This filtrate will support growth of the mutants of Groups I, II and IV but produced no activation of 47313. A preliminary analysis of the active fraction suggests that the activity may be correlated with a ketonic acid. The same substance failed to produce a response in either of the remaining mutants of Group III, i.e., 5936 and 66108.

5936 resembles 47313 but is less pH and temperature sensitive. A profuse grower on minimal medium once it has "adapted" it could nonetheless be recovered quite readily by transfers from old cultures or with dilute inocula. Aside from leucine its most pronounced activation was obtained in the presence of dl-valine (Figure 10). Assuming the ability to grow on the minimal medium might have been a function of the amount of leucine stored in conidia, cultures of 5936 were maintained in growth tubes of increasing concentrations of leucine. Inocula from these cultures and from the minimal controls showed no demonstrable difference in the rate of "adaptation".

The third of the Group III mutants, 66108, is less exacting in its requirements than the others. It grows on minimal medium at a low pH and at 35° C. and is regarded as a leucine mutant only because the most rapid response is to this amino acid. l(-)-Proline, dl-valine and l(+)-lysine gave delayed stiumulation. A brief summary of these three mutants is found in Table X.

FIGURE 10

GROWTH RATES OF 5936 ON
1(-)-LEUCINE, dl-VALINE AND MINIMAL MEDIUM

Sucrose and agar, 2%; temperature, 25° C.

FIGURE 11

GROWTH RATES OF 37501 ON
1(-)-LEUCINE AND MINIMAL MEDIUM

Upper curve obtained with 100 gamma 1(-)-leucine/ml.; lower curve, minimal medium. Agar and sucrose, 2%; temperature, 25° C.

FIGURE 10

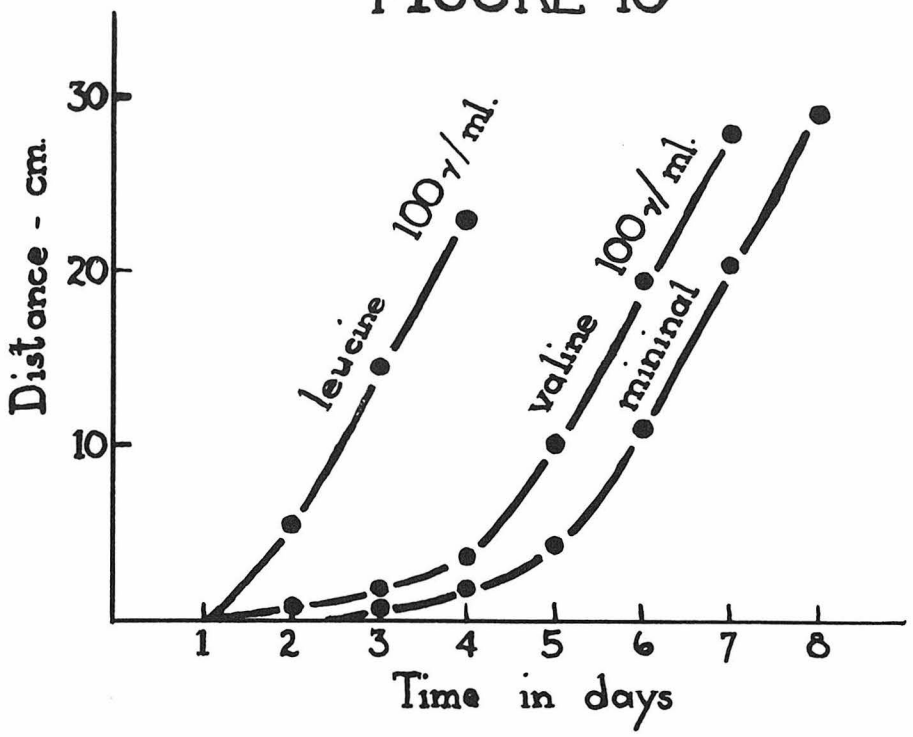


FIGURE 11

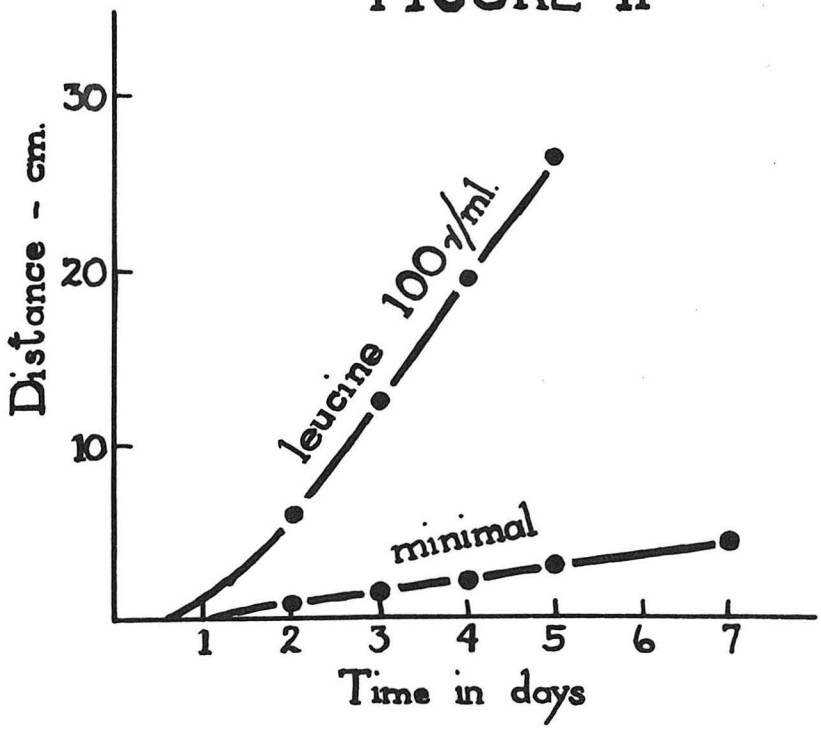


TABLE X

GROWTH RESPONSE OF GROUP III
(Large Inocula)

Stock No.	Minimal 25° C.	Minimal 35° C.	Minimal pH 3.5	Leucine 35° C.	Leucine pH 3.5
66108	+	±	±	+	+
5936	+	±	±	+	+
47313	+	-	-	+	+

A negative response is indicated as -.
A delayed response is indicated as ±.
A positive response is indicated as +.

GROUP IV

37501

The fourth category of growth response is the type exemplified in 37501.

As in the case of Group III the requirement is noted most clearly by using growth tubes because some growth occurs on the minimal medium. The response differs, however, in that the rate of growth on minimal did not equal that on "adequate" leucine concentrations nor did it show a continuous acceleration (Figure 11). The maximum rate of growth on leucine fortified media (2.8 mm./hr.) did not approximate that of a wild type strain (4.0 mm./hr.) on the same medium. This rate was obtained in a concentration of 50 gamma/ml. with no evidence of an

increase with concentrations up to 200 gamma/ml. Growth on minimal medium could be eliminated if the strain was cultured at 35° C.

No growth on minimal medium at 25° C. was obtained if the inoculum used was taken from a stock cultured at 35° C., whereas conidia from cultures maintained at 20° C. germinated and grew on minimal medium at 25° C. This temperature effect was demonstrated most clearly by the following experiment. A growth tube containing minimal agar medium was inoculated with 37501 and maintained at 20° C. After the mycelium had progressed 10 mm. from the point of inoculation, the tube was marked at this point and transferred to 35° C. Shortly thereafter the growth stopped and remained in a static condition. Three days later when returned to 20° C. the mycelial progression was resumed.

From the above results with growth tubes mycelial dry weight values might be predicted. Unless small amounts of leucine are employed the final dry weights should be limited by some factor other than the amino acid. With 200 mg. of sucrose and 10 ml. of media the dry weights indicated by Table XI were obtained after seven days at 25° C.

Of interest is the fact that although the average mycelial weight supported by 1 mg. of leucine was greater than that obtained with 33757 under the same conditions, 33757 produced more mycelia than 37501 with 2 or 4 mg. of leucine. This suggests the possibility of an additional biochemical

TABLE XI

l(-)-Leucine (mg.)	Dry Weight (mg.)
1	44.1
	46.0
	42.2
2	42.4
	45.6
4	47.3
	45.1
	46.5

requirement or an impaired utilization of some constituent in the minimal medium.

The original 37501 strain also showed a pH sensitivity. With an increased acidity no growth occurred in the media lacking leucine. Advantage was taken of this condition to test the activity of leucic acid and synthetic leucine. Using media buffered at pH 4.3 no significant difference in the growth was obtained with equimolar amounts of dl-leucine, calcium leucate and l(-)-leucine.

Like the mutant strains of Groups I and II 37501 showed an increase in growth with added isovaleric acid or isovaleraldehyde although the increase (5%) was lower.

DOUBLE MUTANTS

It is possible by genetic means to "synthesize" Neurospora stocks which will have dual requirements because of the presence of two mutated loci. Similarly double mutant stocks can be produced by crossing two different mutants each having a similar biochemical requirement. (See Genetics section.) The exogenous requirements of such "doubles" may be affected by the sequence of the metabolic steps blocked. For example, if the two steps in the reaction A $\rightleftharpoons$ B $\rightleftharpoons$ C are governed by two independent genes, a block in either or both might lead to the same growth response to C. A block between either A and B or B and C should result in the accumulation of B, if sufficient C is present. The double mutant would eliminate this posibility since B could not be formed by either reaction. If B, or a product of B, acts as an inhibitor, the growth of the double mutant may surpass that of either single; if B is stimulatory, the double may have a decreased response as compared to the singles.

A useful application of double mutants may be found in the combination of a strain which possesses an "absolute" requirement and one whose growth response is of the type exemplified by Group III or IV. Here the reactions might be indicated as above, except that there may be two alternate routes leading from B to C each of which may be gene controlled (A $\overset{1}{\rightleftharpoons}$ B $\overset{2}{\underset{3}{\rightleftharpoons}}$ C). A mutant blocking reaction 1 would be able to grow on B or C, one blocking 2 or 3 would be able to grow on

A, B or C, although the response to A and B may be diminished. A double mutant 1, 2 or 1,3 would grow in the presence of C and to a limited extent in B. Other examples might be postulated along similar lines of reasoning.

A few of the leucineless strains have been combined genetically giving double mutants.

8839 and 33757 are alike in that each has a fundamental "absolute" requirement. The double mutant 8839, 33757 was similar in this respect except that the amount of dry weight obtained on l(-)-leucine was somewhat less than that of 33757 singly. On the other hand when 5936 (Group III) was combined with either 33757 or 8839 the amount of growth exceeded that expected of the single component. Similarly 47313, also of Group III, in combination with 33757 caused an increase in weight.

Of interest in connection with the biochemistry of Group III was the observation that in the course of three days, double mutants involving 66108, 47313 or 5936 with 33757 or 8839 did not grow in the presence of dl-leucic acid. The conditions for testing the activity of the hydroxy acid involved the addition of stimulatory amounts of leucine or inoculation with mycelia (see above). If leucic acid activity is lacking, the amount of growth will reflect the amount of leucine only. However with utilization one obtains a summation of the activity of leucine and leucic acid. The lack of a three day response with the above double mutants is

significant in view of the fact that the single mutants 33757 or 8839 showed an increase in this amount of time.

The filtrate of a pyridoxineless mutant may likewise provide support to the contention that Group III mutants partially lack an amination mechanism. This filtrate supported the growth of Groups I, II and IV mutants but gave a delayed test with those of Group III. The properties of the active fraction indicate the presence of a ketonic acid. The following double mutants: 33757, 47313; 33757, 66108; 8839, 47313; 8839, 66108 and 37501, 47313 showed a negative growth response to the filtrate. Each of these stocks contains one mutant allele of the Group III type. The enhanced response of some of the "doubles" may point to a situation as postulated above, namely, that a block exists on each side of a ketonic acid, hence this compound could not accumulate in the double mutant stock. The single mutants could form this ketonic acid either by leucine degradation or an impaired amination. The keto acid, or its breakdown products, would constitute the inhibitor in this instance. D. Bonner (1946) has described a case of a ketonic acid inhibition in the isoleucine-valine synthesis, a situation which may find analogy here.

Discussion

The evaluation of the role of these mutants in terms of the biosynthesis of leucine is somewhat hindered by the fragmentary nature of the information regarding leucine metabolism in other forms. The following constitutes a summary of the published observations relative to the biochemistry of this amino acid.

l(-)-Leucine is a necessary growth factor for man (Rose, et al., 1943), the growing rat (Womack and Rose, 1936), the mouse (Bauer and Berg, 1943), the chick (Hegsted, 1944) as well as several Lactobacilli. (For references see Dunn, et al., 1947).

Rose (1937) further demonstrated that the l(-)-leucine requirement of the rat could be replaced by the corresponding keto acid as well as the hydroxy acid analogue. The keto acid substitution might be expected since Embden and Schmitz (1912) obtained leucine from surviving dog liver which had been perfused with alpha ketoisocaproic acid and ammonia. Since leucic acid can be converted into alpha ketoisocaproic acid by the action of l-hydroxy acid oxidase (Blanchard, et al., 1945), a mechanism is provided for the utilization of the hydroxy analogue.

Although the rat and mouse are unable to utilize the unnatural isomer of leucine for growth, Ratner, et al. (1940) were able to demonstrate the conversion of the d(+) form to the l(-) form in mature rat tissue. They suppose

that this change proceeds via a symmetrical intermediate, presumably alpha ketoisocaproic acid. Such a conversion is made feasible by d-amino acid oxidase, an enzyme which readily transforms d(+)-leucine to the symmetrical ketonic acid (Krebs, 1935).

Other enzyme systems which can link leucine and the ketonic acid are the transaminases of Braunshtein and Kritzman (1937). In vitro studies (Braunshtein and Asarkh, 1945) showed that the rate of conversion of leucine was rather slow as compared with other amino acids. The inverse is true of the amino oxidase systems.

Lyman, et al. (1947) have attempted to replace the leucine requirement of a bacterium with isoamylamine and carbon dioxide. Their results showed no demonstrable effect.

The initial steps in the degradation of leucine conform to the general course postulated for the typical amino acid. The ketonic acid has been identified as a primary oxidation product by Krebs (1935). Arai (1921) has described the origin of leucic acid in cultures of Bacillus proteus which were grown in the presence of glycerol and leucine. The same analogue was isolated by Schmidt, et al. (1924) from Bacillus granulobacter pectinovorum cultures and the authors assume that the leucic acid arose from the leucine present in the zein of the fermenting corn mash. In no case, however, has it been shown that leucic acid arises by a hydrolytic deamination of leucine and it is supposed that the hydroxy compound

represents reduced keto acid.

By decarboxylation leucine will yield isoamlyamine as was shown by Arai (l.c.). B. proteus which effects this conversion apparently possesses a decarboxylase as well as a deaminase which will act upon leucine.

Both isoamylamine and alpha ketoisocaproic acid may be converted into isovaleric acid, the first by the action of amine oxidase (Pugh and Quastel, 1937), the second by decarboxylation (Harden, 1932). An intermediate in each case might be isovaleraldehyde, a compound which could act as a hydrogen acceptor to form isoamyl alcohol. Thus, one could account for the formation of isoamyl alcohol from leucine as found by Lampitt (1919) and Yamada (1935) in fermenting yeast cultures.

Bloch (1944) has lent further support to the generally accepted idea that isovaleric acid represents an intermediate in the catabolism of leucine.

Although it has been established that leucine is one of the strongly ketogenic compounds (Embden, et al., 1906; Edson, 1935), the final steps leading to the formation of the acetone bodies are obscure.

It should be mentioned that the accumulation of at least one of these intermediates, isoamylamine, may result in the interference with glycolytic systems (Quastel and Wheatly, 1933). d(+)-Leucine can exert an inhibitory effect in L. arabinosa (Fox, et al., 1944). These authors suggest that the

effect is on the utilization of the natural isomer.

The inclusion of the Neurospora leucineless mutants on a chart (Figure 12) summarizing the above data is not possible at the present time. It is possible to replace the l(-)-leucine requirement of all of these strains with dl-leucine but not with d(+)-leucine. Prior to the utilization of the unnatural isomer some l(-)-leucine must be added to the culture. If the inversion is brought about by the intervention of d-amino acid oxidase, the situation could be similar to that described by Edlbacher and Wiss (1945), who reported that l-amino acids activate d-amino oxidases when the latter are present in low concentrations.

The activity of the keto acid analogue indicates that this compound is one precursor of leucine in Neurospora. Because the response to the hydroxy acid analogue requires "adaptation" prior to its utilization it is unlikely that this compound represents a true intermediate in the formation of leucine, but that it can be utilized if oxidized to the keto acid. From the evidence gained from a study of double mutants, the Group III strains represent a genetic interference with the amination of the keto acid.

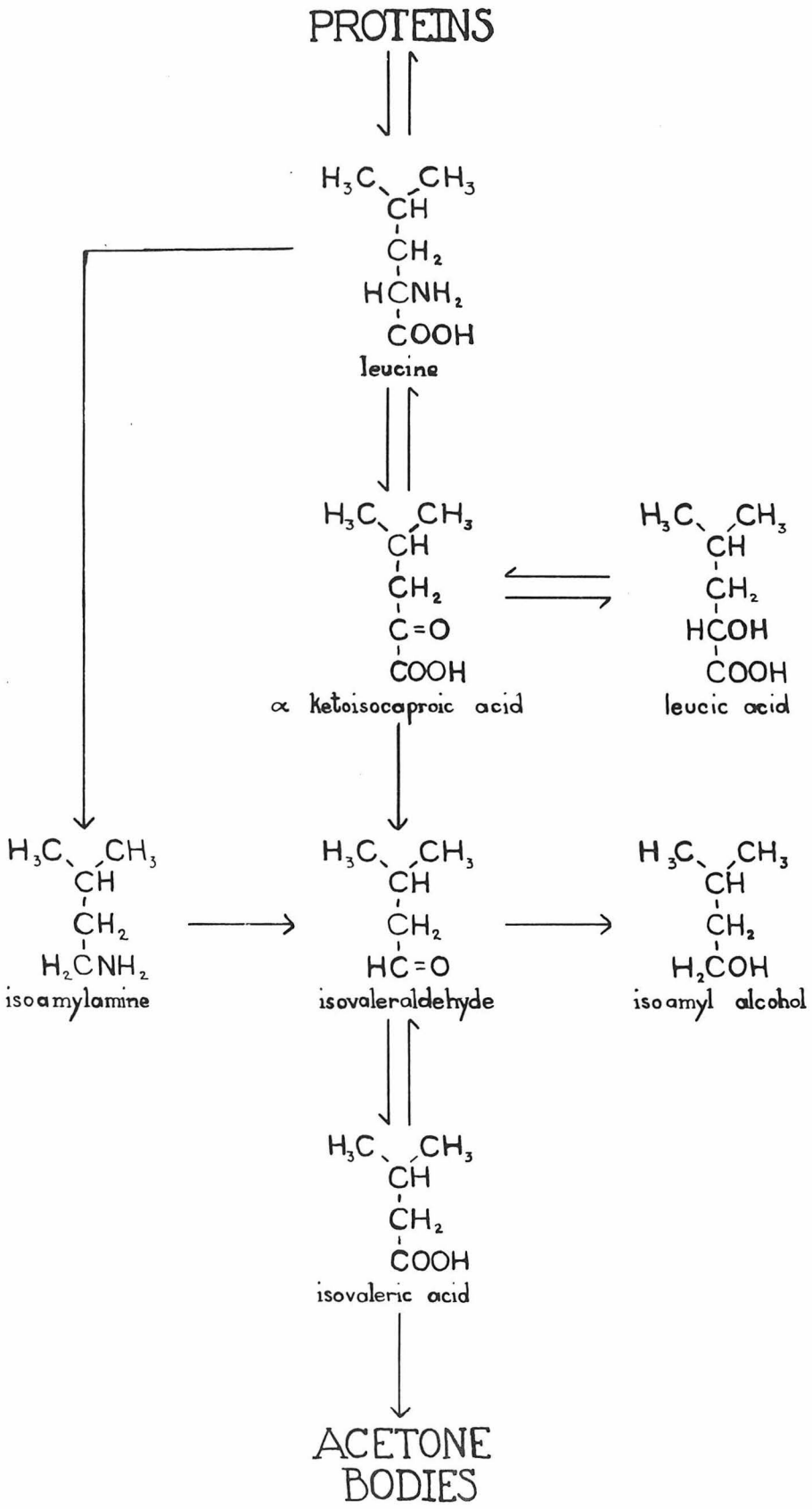
The increase in weight resulting from the addition of the five carbon compounds--isovaleric acid, isovaleraldehyde and isoamyl alcohol--is difficult to account for on the basis of a direct conversion into leucine. There was no experiment which indicated that any of the three were utilized in the

FIGURE 12

SCHEMATIC REPRESENTATION OF THE
METABOLISM OF LEUCINE
IN VARIOUS FORMS

See text for references.

FIGURE 12.


PROTEINS
leucine
α ketoisocaproic acid
leucic acid
isoamylamine
isovaleraldehyde
isoamyl alcohol
isovaleric acid
ACETONE BODIES

absence of leucine. It would seem more likely that they lead to the formation of some compound other than leucine and that the effect is supplementary. It is equally feasible that the activity is the result of a leucine sparing action resulting in a more complete utilization of the leucine initially present in the medium. In support of this contention are the following: (1) the effect of isovaleric acid or isovaleraldehyde was proportional to the amount of leucine present and (2) the addition of these compounds after the cessation of growth, i.e., leucine depletion, eliminated their effect.

The mycelial weight increase with excess sugar seemingly represents a classical type of the sparing of carbohydrates (Kendall, 1922). Where the sucrose was decreased leucine destruction was apparent and the effect diminished with a decrease in the supplied leucine. However, this explanation requires additional qualifications to account for weight increases where the sugar was added after the maximum weight had been attained. A second interpretation is that "excess" sugar leads to the formation of reserves of carbohydrates or other substances which by their incorporation in the mycelium become included in dry weight determinations.

Finally it is possible that some sugars or their derivatives may lead to the formation of leucine by reactions other than those altered by the mutations. Factors which may have some bearing on any interpretation are the lack of increase with xylose and the effects of prolonged heating

of aqueous solutions of glucose and sucrose.

The effects of other amino acids, especially tyrosine, dl-phenylalanine and dl-norleucine, seem to offer no qualitative method by which these mutants may be further segregated. In general, the inhibition was in proportion to the rate of growth of the strain studied; the lower the growth rate, the greater the relative inhibition. Unless the inhibition was a complete one, growth in liquid culture would eventually reach a normal value indicating that the inhibiting effect was neutralized in some fashion as growth proceeded.

The growth on minimal medium of mutants of Groups III and IV was classified as a partial requirement for leucine. Similar "partial" requirements have been observed in several of the Neurospora mutants: isoleucineless (Bonner, et al., 1943), cholineless (Horowitz, et al., 1945), riboflavinless (Mitchell and Houlahan, 1946), pyridoxineless (Stokes, et al., 1943) and arginineless (Srb and Horowitz, 1944). The changes here are not permanent ones in the sense of back mutations but are transitory adaptations to growth on minimal medium. With the leucineless mutants the effect of leucine is primarily a stimulatory one. To decrease the possibility of growth on minimal medium one can employ the following conditions for the strain indicated:

1. Small inocula--66108, 47313, 5936, 37501.
2. Inocula from old cultures--66108, 47313, 5936, 37501.
3. Increase of the temperature--47313, 5936, 37501.
4. Decrease of the pH--47313, 5936, 37501.
5. Inocula from cultures maintained at 35° C.--37501.

Several interpretations for such "partial requirements may be postulated:

1. There may be two pathways for the synthesis of the compound, each of which is operative at a different pH or temperature.
2. Two sets of enzymes may control the reaction; each set subject to the influence of different environmental conditions.
3. The required substance may act in an autocatalytic system.
4. A step leading to the formation of the required compound may under certain conditions, proceed spontaneously as well as by the intervention of an enzyme system.

The Group III mutants could be explained on the basis of any of the schemes. For 37501 the last scheme seems most applicable on the basis of the data on hand. The choice of one of these or other interpretations awaits further experimentation.

In the following section evidence will be presented in an attempt to correlate physiological and genetic similarities or differences.

GENETICS

Essential to the concept that a gene controls a single biochemical reaction is the proof that a given stock is differentiated by a single mutant gene. On theoretical grounds differences in one gene only could rarely be achieved since the parental stocks doubtless differ by many genetic traits, also a "natural" mutation rate will continually give rise to variations within all stocks. However, with respect to a given growth requirement it is possible to demonstrate different single gene mutations with a reasonable degree of certainty.

If a culture is differentiated by the mutation of a single locus, a cross of this stock by a wild type strain should give equal numbers of wild type and mutant ascospores. Since it is possible in Neurospora to recover all four of the meiotic products, a more precise demonstration of a one-to-one ratio can be obtained by an analysis of the eight spores of the ascus. The techniques and methods of analysis have been described in several papers. (Dodge, 1927; Lindegren, 1942.)

To establish the similarity, or difference, between two stocks having the same nutritional requirement two general methods are available. The first involves the crossing of the two stocks and the analysis of the progeny. The second consists of the establishment of a heterokaryon between the

two strains involved. (Beadle and Coonradt, 1944) This method presupposes in the event that the two genetic blocks are dissimilar, each of the stocks contains the factor (gene) absent in the other and thus, in the heterokaryotic hyphae internuclear combinations result in the presence of all of the wild type genes.

Proof of genetic dissimilarity by heterokaryon growth is valid only if each of the strains involved do not "accelerate", or mutate, to wild type. Further the lack of growth does not establish the identity of two strains since there may be factors which prevent effective fusion or subsequent growth.

Establishment of heterokaryons was accomplished with the leucine mutants in some cases by mixing the conidia of the two strains in low concentrations of leucine, thus insuring germination and some growth. Heterokaryons were evident by growth more profuse than in either of the controls.

33757 formed a heterokaryon with 8839 stocks and 37501 stocks, but no growth occurred with mixtures of 33757 with 46409 or 70311. With the above mentioned exception no evidence of heterokaryon formation was obtained with 37501 in combination with any of the mutants of Groups I or II. Mutants of Group III cannot be tested in this fashion because they will grow at a wild type rate after stimulation with leucine. That some of the negative results may become positive under the proper conditions is an unproved assumption.

A priori one would expect trikaryon formation with three double mutant stocks of the following composition: AB/BC/CA since the wild type factor for C would be present in the AB stock, that for A in the BC stock, and the B factor in the CA stock. This was attained with the following leucine stock combinations: 33757, 5936 / 5936, 8839 / 8839, 33757.

Also of interest was the observation that dikaryons, involving 33757 as one member of the pair, would frequently show a decelerating growth rate on minimal agar growth tubes. This phenomenon was not observed in the other cases noted above.

Genetic analyses employed in Neurospora have been applied to many of the biochemical mutants in this organism. A character differentiated by a single gene should be found consistently in two of the four meiotic products. This implies that a mutant stock crossed to a non-homologous strain will yield asci with four mutant spores and four non-mutant spores. A measure of the genetic map distance from the centromere will be found in the arrangement of the spore pairs. If the four end spores at either one or the other end of the ascus show similar characteristics, it is assumed that the gene has segregrated with the centromere at the first meiotic division. Any arrangement showing an alternation of pairs is indicative of a crossover between that gene and the centromere.

Proof of genetic dissimilarity between two stocks

is obtained when an ascus produced from a cross of two such stocks shows four mutant as well as four wild type spores. Additional confirmation is provided when an ascus containing eight mutant spores is derived from an outcross of any one of the four mutant spores of the F1. If the two genes invoked are linked, it may become necessary to make such an analysis on the basis of a six mutant spore pattern instead of four as in the first case. The confirmation in this case requires the outcrossing of each of the three non-wild type pairs because one is the double mutant.

A disturbance in the regular segregation of chromosomes may give rise to asci which are atypical in that the full complement of eight ascospores do not develop. For example, duplication-deficiency types expected from reciprocal translocation crosses may result in only four mature ascospores. Also, by analogy with other organisms chromosomal inversion and non-disjunctional types should in some instances give rise to aberrant ascus types. In such cases the tests for single gene segregation will become more involved.

The genetic analysis of the leucineless mutants was made by dissection of the ascospores from individual asci. In most cases the isolated ascospores were transferred to tubes containing the minimal medium fortified with l-leucine (.01%). After spore activation and growth the cultures were tested for the ability to grow on minimal medium. It is apparent that crosses involving mutants of other biochemical

characters (e.g., adenineless) a second supplement must be added to the isolation medium. Special methods for particular strains were necessary in some cases and will be described in the appropriate section.

33757

Crosses made with 33757 as one parent displayed in general no evidence that the mutant interferred with a normal meiotic picture. Rarely, however, could 33757 be used as the protoperthecial parent with any degree of success. Few fertile perithecia were produced. It is known that l(-)-leucine seems to somewhat inhibit the formation of fruiting bodies regardless of the strain used (Westergaard and Mitchell, 1947). The enrichment with leucine of media upon which the leucineless strains are crossed may act as a detriment in this respect as well as a requirement.

A compilation of the crosses involving 33757 listed in the Appendix shows that the mutant segregated in the first meiotic division in 107 asci, and second division segregrations were found in 22 cases. On the basis of these data the locus of this mutant is about 8.5 map units from the centromere. To relate the gene to one linkage group a series

of crosses were made with other mutants known to be fairly close to the centromere. Coincidental with the analysis of the progeny from these crosses the mating types were determined in 49 cases. Of these 21 asci were found of the parental type; 15 asci represented single crossovers between 33757 and "sex" if the two genes were linked; and on the same basis 13 asci could be classified as double crossovers. It seems highly improbable that the two factors are on the same chromosome. Using a similar type of analysis the following shows the recombination classes of some of the crosses.

TABLE XII

Mutant Involved	Recombinations on Basis of Possible Linkage		
	Noncrossovers	Single Crossovers	Double Crossovers
4637	13	6	3
517	2	3	0
1292	1	5	3
3254	8	2	10
Peach	10	10	2
5801	40	4	0
8839	14	36	12
47313	3	4	0

Of the above the only instance which clearly shows linkage is the one involving 5801. The character, 5801, is determined by a gene which rarely shows second division segregation indicating that this gene is close to the centromere. The data reported here places 33757 about 4.5 map

units from 5801 and probably both genes are located on the same arm. Hungate (1946) has presented more extensive data with the same conclusions.

The last two cases on the above table involve crosses with two other leucineless stocks, 8839 and 47313. As mentioned previously 8839 and 33757 readily form heterokaryons, hence would be expected to show a genetic difference. The formation of a heterokaryon facilitated the analysis of the progeny from crosses involving the two genes since the isolates could be tested against each parental strain. Routinely tests were made in small tubes containing inocula from the isolate and either 8839 or 33757. A loopful of a sterile leucine solution was added to insure germination. As expected the single mutants grew well on the minimal media in the presence of the dissimilar parental type; the double mutants showed no growth.

8839 not only appears as a different gene but in all probability is of a different linkage group. 47313 is easily distinguished from 33757 by its ability to grow on the minimal medium at 25° C. The only difficulty arises in distinguishing between the types 47313, 33757 and 33757 alone. This was overcome by testing the unknown progeny for the ability to grow on the pyridoxineless filtrate mentioned previously. 33757 grows in such media; 47313 gives weak growth. The double mutant did not grow in either this medium or minimal. That this type represented the double mutant was verified by crossing such an isolate to wild type. The progeny clearly

segregated for the two genes 47313 and 33757. Although the data are insufficient to rule out the possibility of linkage between these two genes, the probability is decreased by the strong indication that 47313 belongs to the "sex"-albino linkage group.

With reference to other leucineless mutants no clear cut data are available to lend themselves to the above type of analysis. 66108 was crossed to 33757, 4637 but since most of the asci were incomplete, the ascospores produced were isolated in a random fashion. Of the fourteen spores which germinated four gave a positive test on minimal. Three of these were albino the other wild type in coloration. The remaining ten spores were tested on the pyridoxineless filtrate. Seven cultures, all albino, grew, and the other three, wild type in color, showed a negative response. The last three by analogy with 33757, 47313 were probably the double mutant 33757, 66108.

Incomplete asci likewise resulted from crosses of 33757 X 5936. Those asci which contained eight mature spores seldom were capable of being analyzed since in no case did all of the spores germinate. Asci with six and four mature spores were common but again the lack of germination prevented an accurate evaluation of the data. Several cultures were obtained which would not form heterokaryons with 33757 and did not grow on minimal medium. Such cultures were presumed to be a double mutant. At this time the biochemical test

analagous to those used with 47313 and 66108 was not available but the few cases tested showed no growth on leucic acid, suggesting that 33757 and 5936 were present in the same stock.

In one case 33757 was successfully crossed to 70311. From the perithecia five asci were dissected which showed 100% germination. One of these showed eight leucine requiring spores. The remaining four possessed one wild type spore pair in each ascus. These results indicate a difference between 70311 and 33757 since no wild type spores should be found if the two genes are at the same locus unless a back mutation has occurred in one of the stocks. A back mutation would be expected to give rise to two wild type spore pairs. No tests have been devised to classify the progeny since heterokaryon formation has not been demonstrated between these two strains and no striking biochemical differences has been noted.

The other leucineless mutants mentioned in this paper, 46409 and 37501, have not as yet given a fertile cross with 33757. 37501 forms a heterokaryon with 33757, hence would be expected to differ genetically. 46409 has not been tested genetically to any degree, therefore, no conclusions can be made with reference to its relationship with 33757.

70311

When 70311 was crossed to wild type (25aH2) and the resulting ascospores isolated on leucine fortified media, an analysis of the ascus types was made difficult by the lack of germination in some of the asci. Since the failure to germinate occurred in pairs of ascospores, it was felt that the 70311 stock may have carried some factor which would not manifest itself on either leucine or minimal medium. The cross was made again and this time the ascospores were transferred to "complete" medium. Again pairs of spores failed to germinate or grow; in addition two asci contained six spores which failed to grow on minimal medium but which responded to leucine to some extent. From these data it is not possible to interpret the leucine requirement of 70311 on the basis of a single gene mutation. Further genetic studies are necessary to clarify the meaning of the above results. A summary of the above crosses is indicated below.

TABLE XIII

Spore Pairs			
One	Two	Three	Four
ISOLATED ON LEUCINE FORTIFIED MEDIUM			
-	-	n.g.	+
+	-	+	n.g.
-	-	+	+
+	-	-	+
ISOLATED ON COMPLETE MEDIUM			
n.g.	-	-	-
+	+	-	-
+	+	-	-
-	-	-	n.g.

n.g. = no germination.
- = growth on leucine but not minimal medium.
+ = growth on minimal medium.

46409

No genetic data are available on 46409 except two asci dissected from the cross 46409A X 5801a. The following spore types were obtained.

TABLE XIV

Spore Pairs			
One	Two	Three	Four
58 +	58 +	+ 46	+ 46
58 46	58 +	+ 46	+ +

58 = 5801 (morphological).
46 = 46409 (leucine).
\- = Wild type allele of mutant in corresponding column.

The following matings were unsuccessful: 33757a X 46409A and 70311a X 46409A. When 46409A was crossed to 47313a asci were produced which contained various assortments of aborted spores.

8839

Strain 8839 shows a single gene segregation for its leucine requirement. Of the 88 asci from crosses involving 8839 reported here, 43 showed a first division segregation and 45, a second division. The locus of the gene is thus about 25.5 map units from the centromere.

The 8839 X 33757 crosses have been discussed in connection with 33757. Because of the lability of 8839 it is difficult to be certain that the stock which has been successfully crossed is in reality the mutant or the reverted phase. The mating 8839 X 33757 was made by growing each strain from the opposite ends of a growth tube containing leucine fortified "corn meal agar". In this way the rate of growth could be followed to the point of fusion of the two mutants and constituted some assurance that each remained unreverted immediately prior to their mating. The perithecia which formed at the point of hyphal fusion were dissected and the ascus contents analysed in the usual fashion. The double mutants obtained from this cross were used for subsequent crosses of 8839 since the combination 33757, 8839 remained unreverted.

Crosses with "marker" genes gave the following results. No evidence of linkage is obvious from these data.

TABLE XV

Mutant Involved	Recombinations on basis of Possible Linkage		
	Noncrossovers	Single Crossovers	Double Crossovers
"Sex"	5	26	12
4637	5	9	5
5801	2	7	4
Peach	2	16	4
517	1	3	1
3254	5	12	3
1928	1	7	1

The only other leucineless mutant to which 8839, 33757 was crossed was 5936. The results were similar to other 5936 crosses in giving mostly aberrant ascus types. By heterokaryon tests some ascospore cultures behaved as 8839, 5936.

An 8839 stock which had reverted was crossed to 47313 and 66108. The results of this cross will be discussed under a separate heading. The indications are that 8839 differs from either 47313 or 66108.

A stock presumed to be reverted was crossed with 37501. The products of five asci from this cross were isolated on two different types of media, one containing leucine, the other lacking this factor. The distal spore was placed on leucine fortified media, the adjacent spore on minimal, the third spore on leucine, etc. Since spore number 1 is genetically identical to spore number 2 it was thought that in this fashion 8839 might become evident even though originally

reverted. None of the spores from 8839 X 37501 which were isolated on the minimal medium produced visible germ tubes. All of those isolated to media containing leucine grew. Two types could be distinguished as (1) those which produced a heavy mycelial growth within two days after germination and gave a positive test on minimal medium when fresh growth was utilized and (2) those which produced a sparse mycelium in the first few days and gave a negative minimal test. In the course of several days type 2 grew similarly to type 1 and gave a positive test on minimal whereas type 1 on aging gave no growth on minimal. The obvious explanation for this type of segregation is that 37501 represents type 1, 8839 is type 2 and reverts to a wild type condition in a few days. With such an analysis the asci would be classified as follows:

TABLE XVI

Ascus	Spore Pairs			
	One	Two	Three	Four
A	+ 88	37 +	+ 88	37 +
B	+ 88	37 +	+ 88	37 +
C	+ 88	+ 88	37 +	37 +
D	+ 88	+ 88	37 +	37 +
E	37 +	37 +	+ 88	+ 88

88 = 8839 (leucine).
37 = 37501 (leucine).
+ = Wild type allele of mutant in corresponding column.

Of immediate interest is the fact that no recombination types are apparent despite two second division segregations in Ascus A and B. The chances of such a

segregation occurring with two genes which are not linked is rather remote.

The crosses 8839 X 70311 and 8839 X 46409 produced no fertile perithecia.

47313

Classification of the progeny from crosses involving 47313 may be complicated by the ability of this mutant to grow in the absence of leucine after having grown on media containing this amino acid. The simplest way to effect such a classification is to isolate the ascospores on minimal medium. Spores bearing the 47313 gene will germinate and produce a few hyphae while the wild type allele produces normal growth. If other biochemical mutants are involved in the cross, the medium must be fortified with the required substance. In case the other mutant is one of the leucineless group, spores may be isolated on both leucine fortified medium and the minimal in such a fashion as to include one member of each spore pair on the two types of media. The analysis of the ascus will then be based on the germination response as well as the usual minimal tests.

Three asci dissected from a 47313a X "EMA" cross

gave first division segregation for both 47313 and "sex" with no recombinations. The data from all crosses reported show that 47313 segregated in the first meiotic division in 22 out of 25 asci, indicating that this gene is located about 6 map units from the centromere. The mating type was determined in 14 of these asci, two were second division segregations. In each case the mating type of 47313 as "a", the same as the parental strain. Thus, 47313 shows linkage with the gene which determines the mating type.

The results from crosses with other leucineless mutants show the following relationships:

TABLE XVII

Mutant Involved	Recombinations on Basis of Possible Linkage		
	Noncrossovers	Single Crossovers	Double Crossovers
33757	3	4	0
37501	0	3	1
8839	3	7	1

Since none of the three leucineless mutants indicated here showed "sex" linkage, it would be anticipated that 47313 would not be found in the same linkage group as 33757, 37501 or 8839. 47313 has been crossed to 46409 and 70311. In the first case virtually all of the asci showed aberrant spore patterns. From the 70311 cross five asci were dissected. One spore pair from one of the asci was wild type. As 70311 may contain an aberration or more than one mutant gene a complete

analysis of the progeny is not possible at this time. On the basis of this evidence it may be suggested that 47313 and 70311 are not referable to the same genetic factor or factors.

5936

As mentioned in the sections referring to the genetics of 33757 and 8839, the types of ascus "patterns" obtained from crosses where one parent is 5936 are suggestive of a chromosomal disturbance. Germination of those spores which were dissected from asci was too poor to permit a coherent analysis.

66108

No genetic data are available on this strain other than those referred to in the 33757 and 8839 sections. The germination is good but the separation from wild type frequently is quite arbitrary.

37501

This stock has been mated to "aurescent", 5801, 47313 and 8839. In these crosses the gene segregated in the first division in 17 asci, in the second, 15. A rough approximation of the distance from the centromere equals 23.4 map units. The results were the following:

TABLE XVIII

Mutant Involved	Recombinations on Basis on Possible Linkage		
	Noncrossovers	Single Crossovers	Double Crossovers
"Sex"	3	9	6
5801	2	1	2
"Aures-cent"	2	12	4

No indication of sex linkage or linkage with 47313 or 5801 is apparent. The situation with respect to 8839 has been discussed above. "Sterility" was obtained in crosses with 70311a and 33757a.

Discussion

From the data which have been presented one can infer that the following stocks are leucineless mutants by virtue of a change at a single locus: 33757, 8839, 47313 and 37501. There is not sufficient evidence to make the same conclusion regarding 46409, 66108, 40311 and 5936. The last two stocks listed may reveal chromosomal rearrangement on further study.

Differences which exist among these stocks are tabulated below together with the evidence for such conclusions:

TABLE XIX

	33757	46409	70311	8839	47313	66108	5936
46409	none						
70311	gen.?	none					
8839	het. & gen.	none	none				
47313	gen.	none	none	gen.			
66108	gen.?	none	none	gen.	none		
5936	gen.	none	none	gen.	none	none	
37501	het. & gen.*	none	none	none	gen.	none	none

* By differences in linkage groups.
het. = Heterokaryon.
gen. = Genetic.

Generally genetic differences are apparent where physiological differences were most noticeable. One outstanding exception is that of the genetic relationship of 8839 and 37501. Although these stocks differ greatly in their physiological characteristics, there is neither heterokaryon or genetic data to indicate that they may be deter-

mined by genes at different loci. Difference between stocks of the same physiological group have not been established except possibly in the case of 33757 and 70311.

ADAPTATIONS

Several cases of adaptation have arisen in the studies concerning the leucineless mutants. As mentioned in the biochemical section the growth response to leucic acid, to d(+)-leucine and to intact proteins appears as adaptive phenomena. In an analogous fashion those mutants of Group III readily develop the ability to grow on minimal medium. The perpetuation of these types is dependent upon growing mycelia; that is, it has been possible to transfer such adaptations using hyphal tips as the inocula but not conidia.

A second type of "adaptation" is found with the strain 33757 and 8839. This adaptation, or reversion, occurs as an abrupt change from a leucine requiring strain to one which can grow in the absence of leucine.

33757

The reversion of 33757 has been studied in some detail by Ryan and Lederberg (1946). These authors found that reversions took place in a certain percentage of their cultures and demonstrated that under their conditions the frequency of reverted cultures increased as the concentration of leucine was diminished. Since the author using the same

strain, 33757, 4637, had not observed such changes in more than 200 cultures, it seemed advisable to reinvestigate the problem in an effort to reconcile the differences found by the two laboratories.

Basically the methods of Ryan and Lederberg are identical to those used by the author; the one notable exception is in the volume of the medium used. Whereas Ryan has used 50 ml. cultures, we have restricted the amount to 10 ml. This difference in volumes may not exert an effect per se, but if in each case 2% sucrose were used, the final total of the sugar would show a five fold variation. Thus, in Ryan's cultures there was available 1 gm. of sucrose, whereas we have used 200 mg. As mentioned before this difference will result in a great variation in the mycelial dry weight produced over a period of seven days.

Extending these observations into the question of "reversion", it has been found that here, too, a difference was noted.

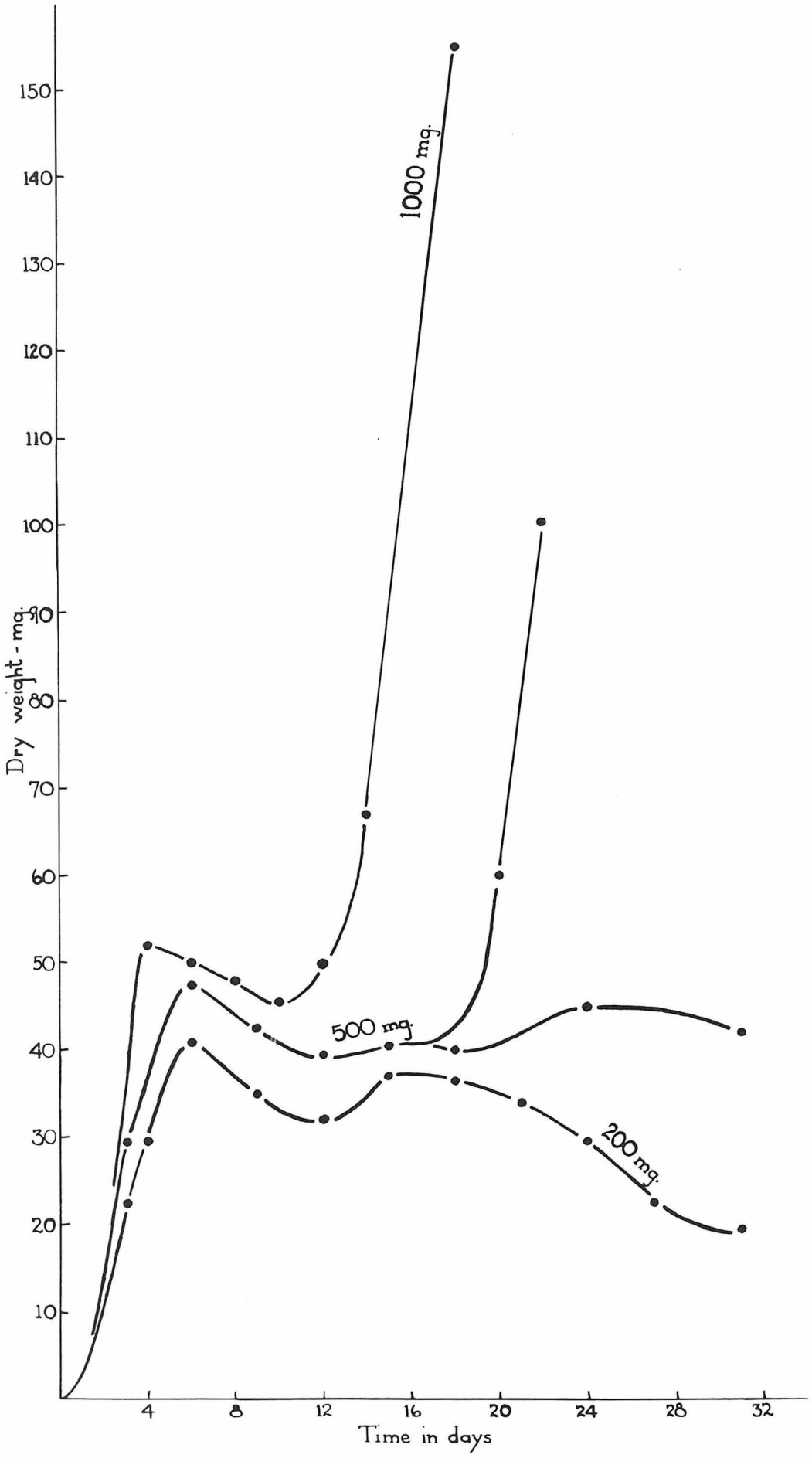
Figure 13 shows the growth curves obtained when 500 and 200 mg. of sucrose were added to 10 ml. of media. The inoculum was made with a suspension of conidia taken from a stock culture grown on 1 mg. l(-)-leucine. After inoculation the flasks were not moved until the weights were to be determined. A sample of mycelium from each culture was tested on minimal medium to determine whether or not it had reverted.

Throughout the course of the experiment no culture containing 200 mg. sucrose showed a positive test for rever-

FIGURE 13

GROWTH CURVE OF STRAIN 33757 AND
THE PRODUCTION OF REVERSIONS

Medium contained 1 mg. l(-)-leucine/10 ml. culture medium, in addition to the amounts of sucrose indicated. Each point represents the average of five mycelial dry weights at the designated time.


150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
Dry weight - mg.
1000 mg.
500 mg.
200 mg.
4
8
12
16
20
24
28
32
Time in days

sion. Of the 500 mg. cultures one reversion was found in a 21 day culture, two in 24 and one each of the 27 and 30 day cultures. Thus, a total of 5 out of 50 cultures, or 10% reversions were obtained. It is believed that this represents a minimum value since the test included but a very small portion of the total mycelial pad and, thus reverted sectors might not be included. All of the cultures which contained 10% sucrose reverted to a wild type condition after 15 days.

If our assumption that the leucine/sucrose ratio is the controlling factor, it should be possible to obtain similar results with reduced amounts of leucine. The following diagram illustrates the percentage of cultures which showed "reverted" mycelia within 30 days. Each average represents from 25 to 50 determinations. The figures are

TABLE XX

l(-)-Leucine (mg.)	Sucrose			
	1000 mg.	500 mg.	200 mg.	100 mg.
1	100%	17%	0	0
0.5	55%	59%	16%	0
0.25	25%	27%	26%	26%

instructive in that they demonstrate how an inverse leucine/reversion relationship could be obtained. Furthermore, it is apparent that where a 10% sucrose solution is utilized the percentage of detected reversions is roughly proportional to the amount of leucine initially present. With

.25 mg. of leucine the percentage of reversions is similar for concentrations of from 1 to 10% sucrose.

As stated previously all of these cultures were not shaken. In a similar experiment the flasks were rotated twice daily for the first 15 days and then allowed to remain stationary for the remaining 15 day period. Of 30 cultures containing 1 mg. l(-)-leucine and 200 mg. sucrose none showed evidence of reversions, whereas 29 cultures containing an initial amount of 500 mg. sucrose reverted to the wild type condition. When rotated continously on a mechanical shaker the effect is even more extreme. The reversions appear in a shorter time interval and, also, can be found occasionally in 1 mg. l(-)-leucine/200 mg. sucrose cultures.

The initial concentration of sucrose has little effect on the frequency of reversions, since with 1 mg. l(-)-leucine and 400 mg. sucrose in 20 ml. Fries medium six of the ten cultures reverted, whereas with 1 mg. l(-)-leucine and 200 mg. of sucrose in 10 ml. none was detected.

Reversions appeared in "non-reverting" cultures (200 mg. sucrose and 1 mg. l(-)-leucine/10 ml.) if supplementary sucrose was added after 10 days, 15 days or 20 days.

To explain these observations one must assume that reversions occur with a fairly regular frequency at some period in the growth of the mutant. The frequency is a function of the amount of mycelium produced or of the number of nuclei present. Once a reversion has occurred, it may

or may not produce a detectable effect on the mycelial weight depending upon the time at which it occurs. If the reversion occurs at a time when the growth promoting capacity of the media is at a minimum, it may escape detection unless the media is supplemented with more sucrose. Thus, the frequency of detection will depend upon the amount of sucrose (or growth supporting substrata) left in the media at the time of reversion. Although the increase in mycelium ceases prior to sucrose depletion there is a progressive decrease in the growth promoting capacity of the media with time.

An alternative hypothesis might be proposed on the basis of the production of a mutagenic substance, or condition where an excess of sucrose is being metabolized. Thus, with larger amounts of mycelium more mutagen is produced. In support of this hypothesis is the observation that the reverted cultures always appear after the mutant growth has reached a maximum and has started to autolyse and further that no reversions have been observed when the mutant is inoculated directly into leucine free media.

8839

The reversion of 8839 cannot be tested in liquid cultures since the frequency of detectable reversions is high even where the sucrose is reduced to 100 mg./1 mg. l(-)-leucine. The change, unlike that of 33757, occurs quite readily in growth tubes and can be studied best under these conditions.

In those cultures which adapt or revert an area of the mycelium appears which eventually overgrows, or outgrows, the unreverted hyphae.

There seemed to be a superficial relationship between the concentration of leucine and the rate of appearance of the reversion. In growth tubes in the lower concentrations, where growth was thin and sporadic, the mutant reverted more readily than it did at the higher concentrations, where growth was heavy and even. That the effect was not the result of the leucine concentration alone was suggested by experiments in which the ratio of leucine to sucrose was varied. Here it was noted that a decrease in the sucrose in tubes, which contained low concentrations of leucine, affected the growth rate slightly but decreased the probability of reversions. In the accompanying table these results are tabulated.

There is no consistent positive correlation between rate of growth and reversion as can be seen by inspection of this table. This would argue against the frequency of reversion increasing with the number of nuclei. As in the case

TABLE XXI

RATE OF GROWTH
(mm./hr.)

Concentration Sucrose %	Concentration l(-)-Leucine (gamma/ml.)	
	12	6
4	3.0*	2.5*
2	3.0*	2.5*
1	2.9*	2.4*
0.5	2.9*	2.4*
0.25	2.7	2.4*

* Reverted after two weeks.

of 33757 similar types of hypothesis may be proposed. Either the existence of a reversion will not be detected if there is a substrate depletion or some substance or condition is produced which stimulates reversion.

Once the reversion has occurred the resultant culture is essentially wild type in its characteristics. It grows on minimal medium at a rate equal to that of wild type, or in many instances in excess of wild type. The inhibitions by dl-phenylalanine and dl-norleucine are not evident, the rate of growth on media containing leucine is the same as wild type on the same media. All subcultures retain these characteristics.

Influence on Other Genes

No effort has been made to determine whether or not changes occur at other loci concomitant to the reversion of 33757. Ryan noted that where the reverting strain is an albino, the albino character remains unaffected and likewise in the few cases tested the mating type is similar to that of the original stock. In addition the reversion of 33757, 5801 resulted in a stock showing the "cauliflower" characteristic of 5801. However, when the reverted 33757, 5801 stock was crossed to wild type, progeny were found which did not resemble the parental types. From one perithecium three asci were dissected each of which segregated for 33757 and 5801. Two asci from another perithecium of the same cross contained no leucine requiring spores. In each case one wild type spore pair was obtained and one pair which carried the 5801 gene. The other two spore pairs were neither wild type nor 5801; both were morphological variants of a small rounded colonial type. No genetic tests have been made using these variants, but one pair would be expected to carry the 5801 gene. The interesting aspect of these results is that with the reversion of 33757 another variant arose, whereas the unreverted nuclei contained no such gene.

Effects of Other Genes

Conversely the effect of other mutants on the frequency of reversion has been followed inafew cases.

The inhibition of the reversion may be brought about by the incorporation of one of the other leucine mutants, 47313, into 33757 stock. The effect is somewhat surprising since 47313 itself readily assumes the characteristics of a wild type stock. The double mutant's leucine requirement is comparatively smaller than that of 33757 and in general the double is slightly "stronger" stock.

In the case of 8839 which is more easily reverted than 33757, the incorporation of 47313 again seems to decrease the reversibility. Although no quantitative tests were made it has been observed that 8839 aconidial stocks seem to be less stable than their conidial counterparts.

Genetics of Reverted Stocks

33757--Ryan and Lederberg (1946) have presented genetic evidence that the reversion of 33757 is a back mutation at that locus. They crossed their reverted strains to the parental leucineless stocks, and in each of the four asci which they reported they obtained four wild type spores. Asci with eight mutant spores which would arise from crosses of the unreverted nuclei of the reverted heterokaryon were not reported.

The existence of mutant nuclei in reverted mycelium was demonstrated by crossing reverted 33757 stocks to wild type or mutant stocks. One case was noted above where 33757, 5801 reverted was crossed to wild type. One perithecium possessed unreverted 33757, the other had the reverted gene. A reverted stock of 33757, 4637 was crossed to wild type. Three asci were dissected from three separate perithecia. All asci segregated for the albino character of 4637 whereas two segregated for leucineless.

8839--The genetic nature of the "reverted" 8839 appears to be superficially similar to the case described by Ryan and Lederberg for 33757 reversion. When two opposite sexes of 8839 were caused to revert and crossed, the resulting cross, though rather "sterile", produced with but few exceptions all wild type spores. Crosses of these wild type spores by wild type likewise resulted in asci with eight

wild type spores. The exceptions mentioned above were found in perithecia along with the reverted, or wild types, and proved to be characteristic of 8839. However, it may be said that of the asci from which eight ascospores were dissected, no case was found in which the original mutant condition was regained.

Upon resumption of this research after a lapse of three years all of the stock cultures of 8839 appeared to be reverted, and an effort was made by the author to regain the original stock from these cultures. 47313a was crossed to the reverted 8839 stock and the ascospores were isolated on medium containing 2% agar, 2% sucrose and .1 mg. l(-)-leucine/ ml. of Fries medium. After germination the resulting cultures were tested on minimal medium. The results are tabulated below:

TABLE XXII

Perithecium	Ascus Number	Minimal Tests of Spore Pairs by Number			
		1-2	3-4	5-6	7-8
A	1	+	+	-	+
	2	+	+	+	+
C	1	+	+	-	+
	2	+	-	+	+
D	1	-	+	+	+
F	1	+	+	+	+
H	1	-	+	+	+
I	1	+	+	+	-
J	1	+	+	-	-
K	1	+	+	-	+

The presence of 47313 was determined by testing the isolates on minimal medium at 35° C. In those asci which gave a positive minimal test with six spores 47313 appeared in two of the six. In ascus A2 47313 was present in spores 5,6,7 and 8, whereas in J, spores 1, 2, 3 and 4 tested as wild type at both 25° and 35° C.

Thus, it appeared probable that every spore which gave a negative minimal response at 25° C. contained the gene 47313. Ordinarily 47313 gives a positive response under these conditions. The leucine requiring isolates (5 and 7) of ascus J were crossed to EMA and produced five + + + - asci and four + + + +. Five asci were dissected from the cross C2-1 X EMA and on early testing three of the isolated spore cultures gave no growth on minimal. A day later all spore cultures grew on the minimal medium. C2-5 X 25aH2 produced all wild type cultures in the five asci analyzed.

Most significant were the crosses involving ascus A2. Four of the spores (A2-5, A2-6, A2-7 and A2-8) gave cultures which were 47313 in their physiological characteristics. A2-5 and A2-7 crossed to 25aH2 (wild type) yielded asci which segregated for 47313. Five asci were analysed in each case. The other four spores of ascus A2 when tested gave a wild type growth response on minimal media. When crossed to wild type (25aH2) A2-2 produced all wild type spores in the four asci dissected. Likewise, the cross A2-4 X 25aH2 gave rise to 32 wild type progeny in four asci.

A2-2 was also crossed to 66108a, a mutant similar to 47313, and gave the following ascus types:

TABLE XXIII

Perithecium	Minimal Tests of Spore Pairs by Number			
	1-2	3-4	5-6	7-8
A	-	+	+	+
B	+	+	+	-
C	+	+	-	+
D	+	-	-	+
E	+	+	+	+

Tests of the above spores revealed that the pairs A-5 and A-6, B-1 and B-2 and C-7 and C-8, and the first four spores of ascus E were similar to 66108 in their characteristics. In ascus D no isolate gave a test indicative of the presence of 66108 alone. In this respect it was similar to ascus J of the original cross.

The simplest interpretation of these data is that those spores which give rise to cultures that will not grow on the minimal media possess either the gene 47313 or 66108, depending upon the particular cross. In addition these cultures contain a modifier which makes the leucine requirement an "absolute" one rather than the "partial" type of 47313 or 66108 alone. The "modifier" appears to segregate as a single gene. This modifier apparently was present in the reverted 8839 stock to which 47313 was crossed. The fact

that 47313, "modifier" resembles 47313, 33757 in its biochemical requirements strongly suggests that the "modifier" may be in reality unreverted 8839 since 33757 and 8839 are very similar in their growth requirements.

Thus, the original reverted culture of 8839 may not have resulted from a back mutation to the wild type condition but rather from a change of a different nature. The change probably does not consist of the mutation of a suppressor since one would expect the suppressor to segregate as frequently in crosses with wild type as it would seem to in crosses involving 47313.

Two other possible interpretations may be offered: (1) that the reversion of 8839 consists of a mutation at the 8839 locus to an allele which is wild type in expression, except in the presence of 47313 or 66108, or (2) that the reversion is a cytoplasmic "adaptation" which persists through a sexual generation but is not active or effective where the gene 47313 is present.

Discussion

Variability of microorganisms has long been subject to many controversial interpretations especially with respect to the genetic implications. Differences in morphology and physiology arising under the influence of environmental conditions have been noted in a variety of forms and have been designated by as many different terms. The literature which has accumulated in this field is summarized and interpreted in several comprehensive reviews (Yudkin, 1938; Stephenson, 1939; Gale, 1943; Dubos, 1945).

Physiological variation as a result of gene mutation is now a well established doctrine largely through the efforts of Beadle and Tatum and their co-investigators. These variations arise in response to the same agents known to produce mutations in the higher forms and their inheritance is typically Mendelian. In Neurospora variability arising in response to cultural changes was described by Went (1901). More recently Emerson and Cushing (1946) have investigated the inheritance of similar types of variation and found some can be interpreted on the basis of mutation and selection. Other types however seem to be non-heritable and are expressed as transient adaptations. Emerson (1945) has suggested that these two types of changes may not represent unrelated phenomena but may be manifestations of a common type of response.

In general, however, there seem to be two types

of changes, the genic and consequently heritable type and the non-genic type which is inherited only in the asexual phases. The latter type is typical of the leucineless mutants of Group III. The variations tend to disappear as the culture ages and require for their preservation an actively growing mycelium. To all appearances the 33757 reversion seems to be a true back mutation, one which brings about the restoration of the original wild type allele.

The change found with 8839 does not seem to fall into either category. Here the reversion is transmitted through the conidia and in a qualified fashion through the ascospores. That it does not represent a true mutation to the wild type allele is evidenced by the reappearance of a mutant condition in certain types of crosses and the lack of appearance in others. In some aspects the situation appears analogous to the production of the "kappa" substance by Paramecium aurelia (Sonneborn, 1945) and the "cytogene" described by Lindegren, et. al. (1944).

Regardless of the exact nature of these changes or reversions, their production and their inheritance, it may be asked whether or not is possible to draw a line between purely cytoplasmic changes and those involving the gene itself. The present study does not pretend to solve any of these questions or the many which arose in the course of experimentation, but it is hoped that it may offer an approach toward these ends.

SUMMARY

Eight mutant strains of Neurospora crassa requiring leucine for growth were investigated. These were arbitrarily divided into four groups on the basis of growth response.

All mutants grow on media containing l(-)-leucine or dl-leucine but not on d(+)-leucine unless small amounts of the natural isomer are added. Groups I, II and IV give a growth response to dl-leucic acid following a short period of adaptation. Groups I and IV yield higher mycelial dry weights when supplementary amounts of isovaleric acid, isovaleraldehyde or isoamyl alcohol are added to leucine-medium. "Excess" of various sugars produce increased weights with the same mutants.

Group III strains may partially block an amination mechanism leding to the formation of leucine. The other strains may involve blocks in the sequence of steps leading to the formation of alpha ketoisocaproic acid.

Strains 33757, 8839, 47313 and 37501 differ from the wild type in that each represents the result of a single gene mutation. Differences between groupings are reflected in the genetic differences except in the case of 8839 and 37501.

Some stocks tend to revert to a wild type condition. Reversions of 33757 and 8839 are detected where the sucrose/leucine ratio is high. Matings of reverted stocks indicate that the reverted 33757 represents a back mutation to the wild type condition. Reverted 8839 may or may not retain its wild type growth habit depending upon the stocks to which it crossed. The accelerated phase of 47313 is not inherited except asexually.

BIBLIOGRAPHY

Arai, M. (1921) Bioch. Zeit. 122:251.

Bauer, C. D. and Berg, C. P. (1943) J. Nutrition 26:51.

Beadle, G. W. (1945) Physiol. Rev. 25:643.

Beadle, G. W. and Coonradt, V. L. (1944) Genetics 29:291.

Beadle, G. W. and Tatum, E. L. (1941) Proc. Natl. Acad. Sci. U. S. 27:499.

Beadle, G. W. and Tatum, E. L. (1945) Amer. Jour. Bot. 32:678.

Blanchard, M., Green, D. E., Nocito, V. and Ratner, S. (1945) J. Biol. Chem. 161:583.

Bloch, K. (1944) J. Biol. Chem. 155:255.

Bonner, D. (1946) J. Biol. Chem. 166:545.

Bonner, D., Tatum, E. L. and Beadle, G. W. (1943) Arch. Biochem. 3:71.

Braunshtein, A. E. and Asarkh, M. (1945) J. Biol. Chem. 157: 421.

Braunshtein, A. E. and Kritzman, M. G. (1937) Enzymologia 2:129.

Dodge, B. O. (1927) Jour. Agric. Res. 35:289.

Dubos, R. J. (1945) in The Bacterial Cell, Harvard Univ. Press.

Dunn, M., Shankman, S., Camein, M. and Block, H. (1947) J. Biol. Chem. 168:1.

Edlbacher, S. and Wiss, O. (1945) Helv. Chim. Acta. 27:1831.

Edson, N. L. (1935) Bioch. Jour. 29:2498.

Embden, G., Solomon, H. and Schmidt, F. (1906) Beitr. Chem. Physiol. u. Path. 8:129.

Embden, G. and Schmitz, E. (1912) Bioch. Zeit. 38:393.

Emerson, S. (1945) Ann. Mo. Bot. Gard. 32:243.

Emerson, S. and Cushing, J. E. (1946) Federation Proc. (Amer. Soc. Biol. Chem.) 3:379.

Fox, S. W., Fling, M. and Bollenback, G. N. (1944) J. Biol. Chem. 155:465.

Gale, F. E. (1943) Bact. Rev. 7:139.

Haldane, J. B. S. (1937) in Perspectives in Biochemistry, Cambridge.

Harden, A. (1932) Alcoholic Fermentation, Longmans, Green & Co.

Hegsted, D. M. (1944) J. Biol. Chem. 156:247.

Hodson, A. Z. and Krueger, G. M. (1947) Archiv. Biochem. 12:435.

Horowitz, N. H. (1944) J. Biol. Chem. 154:141.

Horowitz, N. H., Bonner, D. Houlahan, M. B. (1945) J. Biol. Chem. 159:145.

Horowitz, N. H., Houlahan, M. B., Hungate, M. G. and Wright, B. (1946) Science 104:233.

Hungate, F. P. (1946) Ph. D. Thesis, Stanford Univ.

Kendall, A. I. (1922) J. Inf. Dis. 30:211.

Krebs, A. H. (1935) Bioch. Jour. 29:1620.

Lampitt, L. H. (1919) Biochem. Jour. 13:459.

Lindegren, C. C. (1942) Iowa State Coll. Jour. Sci. 15:271.

Lindegren, C. C. Spiegelman, S. and Lindegren, G. (1944) Proc. Natl. Acad. Sci. U. S. 30:346.

Lyman, C. M., Moseley, O., Wood, S., Butler, B. and Hale, F. (1947) J. Biol. Chem. 168:

Mitchell, H. K. and Houlahan, M. B. (1946) Amer. Jour. Bot. 33:31.

Pugh, C. E. M. and Quastel, J. H. (1937) Biochem. Jour. 31:286.

Quastel, J. H. and Wheatley, A. H. M. (1933) Biochem. Jour. 27:1609.

Ratner, S., Schoenheimer, R. and Rittenberg, D. (1940) J. Biol. Chem. 134:653.

Regnery, D. C. (1944) J. Biol. Chem. 154:151.

Ryan, F. J. (1946) Federation Proc. (Amer. Soc. Biol. Chem.) 3:366.

Ryan, F. J., Beadle, G. W. and Tatum, E. L. (1943) Amer. Jour. Bot. 30:784.

Ryan, F. J. and Brand, E. (1944) J. Biol. Chem. 154:161.

Ryan, F. J. and Lederberg, J. (1946) Proc. Natl. Acad. Sci. U. S. 32:163.

Rose, W. C. (1937) Science 86:289.

Rose, W. C., Haines, W. J., Johnson, J. E., Warner, D. T. (1943) J. Biol. Chem. 148:457.

Schmidt, E. G. Peterson, W. H. and Fred, E. B. (1924) J. Biol. Chem. 61:163.

Sonneborn, T. M. (1945) Ann. Mo. Bot. Gard. 32:213.

Srb, A. M. and Horowitz, N. H. (1944) J. Biol. Chem. 154:129.

Stephenson, M. (1939) in Bacterial Metabolism, Longmans, Green and Co.

Stokes, J. L., Foster, J. W. and Woodward, C. R. (1943) Arch. Biochem. 2:235.

Went, F. A. F. C. (1901) Centralbl. f. Bacteriol., Abteilung 2, 7:591.

Westergaard, M. and Mitchell, H. K. (1947) Amer. Jour. Bot. (In press.)

Womack, M. and Rose, W. C. (1936) Jour. Biol. Chem. 116:381.

Wright, S. (1941) Physiol. Rev. 21:487.

Yamada, M. (1935) Bull. Agric. Chem. Soc. Japan 11:21.

Yudkin, J. (1938) Biol. Rev. (camb) 13:93.

33757 a X 4637 A

SPORE PAIRS				NO. ASCI
ONE	TWO	THREE	FOUR	
33 + a	33 + a	+ al A	+ al A	4
+ al A	+ al A	33 + a	33 + a	4
+ al a	+ al A	33 + A	33 + a	1
33 al A	33 al A	+ + a	+ + a	2
+ + a	+ + a	33 al A	33 al A	1
33 al a	33 + a	+ al A	+ + A	1
+ al A	33 al A	33 + a	+ + a	2
+ al A	33 al a	+ + a	33 + A	1
33 al a	+ al a	33 + A	+ + A	1
			Total	17

SEGREGATION:

33757	1st Division	13
	2nd Division	4
4637	1st Division	16
	2nd Division	1
"Sex"	1st Division	14
	2nd Division	3

RECOMBINATIONS on basis of possible linkage:

33757-4637	Non-crossovers	9
	Single Crossovers	5
	Double Crossovers	3
33757-Sex	Non-crossovers	9
	Single Crossovers	5
	Double Crossovers	3
4637-Sex	Non-crossovers	13
	Single Crossovers	3
	Double Crossovers	1

REMARKS:

Isolated on leucine fortified media.
Germination--75%.

SYMBOLS:

al = 4637 (albino).
33 = 33757 (leucine).
+ = Wild type allele of mutant in corresponding column.
A and a = Mating types.

8839, 4637 A X "crassa a"

SPORE PAIRS				NO. ASCI
ONE	TWO	THREE	FOUR	
+ + a	+ + a	88 al A	88 al A	2
+ al A	+ al A	88 + a	88 + a	3
88 + a	88 + a	+ al A	+ al A	1
88 + a	88 al a	+ + A	+ al A	1
+ + a	+ + A	88 al A	88 al a	1
+ + a	+ al a	88 al A	88 + A	1
88 + a	+ + a	+ al A	88 al A	1
+ al A	88 al A	+ + a	88 + a	1
+ + A	88 al A	88 al a	+ + a	1
88 + A	+ + A	+ al a	88 al a	1
88 al A	+ al A	88 + a	+ + a	1
			Total	14

REMARKS:

Reversion of 8839 was frequent as cultures aged.
Germination--79%.

SYMBOLS:

al = 4637 (albino).
88 = 8839 (leucine).
+ = Wild type allele of mutant in corresponding column.
A and a = Mating types.
"crassa a" = Wild type stock.

8839, 4637 A X "crassa a"
(continued)

SEGREGATION:

8839	1st Division	9
	2nd Division	5
4637	1st Division	11
	2nd Division	3
"Sex"	1st Division	13
	2nd Division	1

RECOMBINATIONS on basis of possible linkage:

8839-4637	Non-crossovers	4
	Single Crossovers	6
	Double Crossovers	4
8839-Sex	Non-crossovers	3
	Single Crossovers	6
	Double Crossovers	5
4637-Sex	Non-crossovers	9
	Single Crossovers	4
	Double Crossovers	1

8839, 4637 A X 33757 a

SPORE PAIRS				NO. ASCI
ONE	TWO	THREE	FOUR	
33 + + a	33 + + a	+ 88 al A	+ 88 al A	1
33 88 + A	33 + + A	+ 88 al a	+ + al a	1
+ + al A	+ 88 al a	33 88 + A	33 + + a	1
+ + al A	+ + al a	33 88 + a	33 88 + A	1
33 + al A	+ 88 al A	33 88 + a	+ + + a	1
			Total	5

REMARKS:

Cross made on leucine fortified media.
Few perithecia.
Leucine mutants classified by heterokaryon tests and the following outcrosses.
Germination--82%.

SYMBOLS:

al = 4637 (albino).
33 = 33757 (leucine).
88 = 8839 (leucine).
+ = Wild type allele of mutant in corresponding column.
A and a = Mating types.

8839, 4637 A X 33757 a
(continued)

SEGREGATION:

33757	1st Division	4
	2nd Division	1
8839	1st Division	2
	2nd Division	3
4637	1st Division	5
	2nd Division	0
"Sex"	1st Division	3
	2nd Division	2

RECOMBINATIONS on basis of possible linkage:

33757-8839	Non-crossovers	1
	Single Crossovers	3
	Double Crossovers	1
33757-4637	Non-crossovers	4
	Single Crossovers	1
	Double Crossovers	0
33757-Sex	Non-crossovers	1
	Single Crossovers	3
	Double Crossovers	1
8839-4637	Non-crossovers	1
	Single Crossovers	3
	Double Crossovers	1
8839-Sex	Non-crossovers	0
	Single Crossovers	4
	Double Crossovers	1
4637-Sex	Non-crossovers	2
	Single Crossovers	2
	Double Crossovers	1

517 A X 8839, 33757 a

SPORE PAIRS				NO. ASCI
ONE	TWO	THREE	FOUR	
+ F +	+ F 88	33 + +	33 + 88	1
+ F 88	+ F 88	33 + +	33 + +	1
33 F +	+ F 88	+ + 88	33 + +	1
33 + 88	+ + 88	+ F +	33 F +	1
+ F +	33 + +	+ + 88	33 F 88	1
			Total	5

SEGREGATION:

33757	1st Division	2
	2nd Division	3
8839	1st Division	3
	2nd Division	2
517	1st Division	4
	2nd Division	1

RECOMBINATIONS on basis of possible linkage:

33757-8839	Non-crossovers	0
	Single Crossovers	4
	Double Crossovers	1
33757-517	Non-crossovers	2
	Single Crossovers	3
	Double Crossovers	0
8839-517	Non-crossovers	1
	Single Crossovers	3
	Double Crossovers	1

REMARKS:

Leucine mutants classified by heterokaryon tests.
Isolated on "complete" media.
Germination--90%.

SYMBOLS:

F = 517 (fat).
33 = 33757 (leucine).
88 = 8839 (leucine).
+ = Wild type allele of mutant in corresponding column.
A and a = Mating types.

1298 a X 8839, 33757 A

SPORE PAIRS				NO. ASCI
ONE	TWO	THREE	FOUR	
+ + +	+ + +	ur 88 33	ur 88 33	1
+ 88 +	+ 88 +	ur + 33	ur + 33	1
+ + 33	+ 88 33	ur + +	ur 88 +	1
ur 88 33	ur + 33	+ + +	+ 88 +	1
ur 88 33	ur + +	+ + 33	+ 88 +	1
ur 88 +	+ 88 +	+ + 33	ur + 33	1
ur 88 +	+ 88 +	ur + 33	+ + 33	1
ur + +	+ + 33	+ 88 33	ur 88 +	1
ur + 33	+ 88 33	+ + +	ur 88 +	1
			Total	9

REMARKS:

Leucine mutants classified by heterokaryon tests.
Isolated on "complete" media.
Germination--98%.

SYMBOLS:

ur = 1298 (uracil).
33 = 33757 (leucine).
88 = 8839 (leucine).
+ = Wild type allele of mutant in corresponding column.
A and a = Mating types.

1298 a X 8839, 33757 A
(continued)

SEGREGATION:

33757	1st Division	7
	2nd Division	2
8839	1st Division	5
	2nd Division	4
1298	1st Division	5
	2nd Division	4

RECOMBINATIONS on basis of possible linkage:

1298-8839	Non-crossovers	1
	Single Crossovers	7
	Double Crossovers	1
1298-33757	Non-crossovers	1
	Single Crossovers	5
	Double Crossovers	3
8839-33757	Non-crossovers	2
	Single Crossovers	4
	Double Crossovers	3

3254 a X 8839, 33757 A

SPORE PAIRS				NO. ASCI
ONE	TWO	THREE	FOUR	
ad + + A	ad + + A	+ 33 88 a	+ 33 88 a	1
+ 33 88 a	+ 33 88 a	ad + + A	ad + + A	1
ad 33 + A	ad 33 + A	+ + 88 a	+ + 88 a	1
+ + 88 a	+ + 88 a	ad 33 + A	ad 33 + A	1
+ + + a	+ + + a	ad 33 88 A	ad 33 88 A	1
+ + + A	+ + + A	ad 33 88 a	ad 33 88 a	1
ad 33 88 a	ad 33 88 a	+ + + A	+ + + A	1
+ 33 88 A	+ 33 + A	ad + + a	ad + 88 a	1
+ 33 88 a	+ 33 + a	ad + + A	ad + 88 A	1
+ 33 + A	+ 33 88 A	ad + 88 a	ad + + a	1
+ + 88 A	+ + + A	ad 33 + a	ad 33 88 a	1
+ + + a	+ + 88 A	ad 33 + A	ad 33 88 a	1
ad + + a	ad + 88 a	+ 33 88 A	+ 33 + A	2
ad + + A	ad + 88 A	+ 33 + a	+ 33 88 a	1
ad 33 88 a	ad 33 + a	+ + + A	+ + 88 A	1
ad 33 88 a	ad 33 + a	+ + 88 A	+ + + A	1
ad 33 + A	ad 33 88 A	+ + 88 a	+ + + a	1
+ + 88 A	+ 33 88 A	ad + + a	ad 33 + a	1
+ 33 88 A	+ + + a	ad + + A	ad 33 88 a	1
			Total	20

SYMBOLS:

ad = 3254 (adenine).
33 = 33757 (leucine).
88 = 8839 (leucine).
+ = Wild type allele of mutant in corresponding column.
A and a = Mating types.

3254 a X 8839, 33757 A
(continued)

SEGREGATION:

33757	1st Division	18
	2nd Division	2
8839	1st Division	8
	2nd Division	12
3254	1st Division	20
	2nd Division	0
"Sex"	1st Division	17
	2nd Division	3

RECOMBINATIONS on basis of possible linkage:

3254-8839	Non-crossovers	5
	Single Crossovers	12
	Double Crossovers	3
3254-33757	Non-crossovers	8
	Single Crossovers	2
	Double Crossovers	10
3254-Sex	Non-crossovers	10
	Single Crossovers	2
	Double Crossovers	8
8839-33757	Non-crossovers	6
	Single Crossovers	12
	Double Crossovers	2
8839-Sex	Non-crossovers	2
	Single Crossovers	12
	Double Crossovers	6
33757-Sex	Non-crossovers	8
	Single Crossovers	3
	Double Crossovers	9

REMARKS:

Leucine mutants classified by heterokaryon tests.
Germination--74%.

HETEROKARYON { 3100 A / 8839, 33757 A } X Peach a

SPORE PAIRS				NO. ASCI
ONE	TWO	THREE	FOUR	
+ P +	+ P +	33 + ȣȣ	33 + ȣȣ	1
+ P ȣȣ	+ P +	33 + ȣȣ	33 + +	1
+ P ȣȣ	+ P +	33 + +	33 + ȣȣ	1
+ P +	+ P ȣȣ	33 + ȣȣ	33 + +	1
33 + + *	33 + ȣȣ*	+ P ȣȣ*	+ P + *	1
33 + + *	33 + +	+ P ȣȣ@	+ P ȣȣ@	1
33 + +	33 + +	+ P ȣȣ	+ P ȣȣ	1
33 + ȣȣ	33 + +	+ P ȣȣ	+ P +	1
33 + +	33 + ȣȣ	+ P +	+ P ȣȣ	2
33 P ȣȣ	33 + ȣȣ	+ P +	+ + +	1
33 P ȣȣ	33 + ȣȣ	+ + +	+ P +	1
33 + ȣȣ	33 P ȣȣ	+ + +	+ P +	2
33 + ȣȣ	33 P +	+ P ȣȣ	+ + +	1
33 + +	33 P +	+ P ȣȣ	+ + ȣȣ	1
+ + +	+ P ȣȣ@	33 + ȣȣ	33 P +	1
+ + ȣȣ@	+ P ȣȣ	33 + +	33 P +	1
+ + ȣȣ*	+ P +	33 + ȣȣ	33 P +	1
33 P + *	+ P ȣȣ*	33 + + *	+ + ȣȣ*	1
+ P ȣȣ	33 + +	33 + +	+ P ȣȣ	1
+ P ȣȣ	33 + +	33 P ȣȣ	+ + +	1
			Total	22

HETEROKARYON { 3100 A / 8839, 33757 A } X Peach a
(continued)

SEGREGATION:

33757	1st Division	19
	2nd Division	3
8839	1st Division	10
	2nd Division	12
Peach	1st Division	11
	2nd Division	11

RECOMBINATIONS on basis of possible linkage:

33757-8839	Non-crossovers	5
	Single Crossovers	13
	Double Crossovers	4
33757-Peach	Non-crossovers	10
	Single Crossovers	10
	Double Crossovers	2
8839-Peach	Non-crossovers	2
	Single Crossovers	16
	Double Crossovers	4

REMARKS:

Number of perithecia containing 33757, 8839 = 22.
Number of perithecia containing 3100 = 2.
Leucine mutants classified by heterokaryon tests.
Germination--89%.

SYMBOLS:

P = Peach.
33 = 33757 (leucine).
88 = 8839 (leucine).
+ = Wild type allele of mutant in corresponding column.
A and a = Mating types.
* At least one member of spore pair germinated without heat activation.
@ Reverted.

5801, "N" a X 33757, 8839 A

SPORE PAIRS				NO. ASCI
ONE	TWO	THREE	FOUR	
+ + + N	33 88 + +	+ 88 58 N	33 + 58 +	1
+ + 58 N	+ 88 58 N	33 + + +	33 88 + +	1
			Total	2

SEGREGATION:

33757	1st Division	1
	2nd Division	1
8839	1st Division	0
	2nd Division	2
5801	1st Division	2
	2nd Division	0
"N"	1st Division	1
	2nd Division	1

RECOMBINATIONS on basis of possible linkage:

33757-8839	Non-crossovers	0
	Single Crossovers	2
	Double Crossovers	0
33757-5801	Non-crossovers	1
	Single Crossovers	1
	Double Crossovers	0
33757-"N"	Non-crossovers	2
	Single Crossovers	0
	Double Crossovers	0
8839-5801	Non-crossovers	0
	Single Crossovers	2
	Double Crossovers	0
8839-"N"	Non-crossovers	0
	Single Crossovers	2
	Double Crossovers	0

REMARKS:

33757-5801, double mutant, used for succeeding linkage tests. Germination--100%.

SYMBOLS:

N = Non-conidial.
58 = 5801 (morphological).
33 = 33757 (leucine).
88 = 8839 (leucine).
+ = Wild type allele of mutant in corresponding column.
A and a = Mating types.

33757, 5801 A X "crassa a"

SPORE PAIRS				NO. ASCI
ONE	TWO	THREE	FOUR	
33 58	33 58	+ +	+ +	20
+ +	+ +	33 58	33 58	19
33 58	+ 58	33 +	+ +	1
33 +	+ +	+ 58	33 58	1
+ +	33 +	33 58	+ 58	1
			Total	42

SEGREGATION:

33757	1st Division	39
	2nd Division	3
5801	1st Division	42
	2nd Division	0

RECOMBINATIONS on basis of possible linkage:

33757-5801	Non-crossovers	39
	Single Crossovers	3
	Double Crossovers	0

REMARKS:

Isolated on leucine fortified media.
Germination--97%.

SYMBOLS:

58 = 5801 (morphological).
33 = 33757 (leucine).
+ = Wild type allele of mutant in corresponding column.
A and a = Mating types.
"crassa a" = Wild type stock.

8839, 5801, "N" A X 25 a

SPORE PAIRS				NO. ASCI
ONE	TWO	THREE	FOUR	
+ 58 N	+ 58 N	88 + +	88 + +	1
+ 58 +	+ 58 N	88 + +	88 + N	1
+ 58 N	+ 58 +	88 + N	88 + +	1
+ 58 +	+ 58 N	88 + N	88 + +	1
88 58 N	88 58 N	+ + +	+ + +	1
88 58 N	88 58 +	+ + +	+ + N	1
+ 58 N	88 58 +	88 + +	+ + N	1
+ 58 +	88 58 +	+ + N	88 + N	1
88 58 N	+ 58 N	88 + +	+ + +	1
88 + N	+ + N	88 58 +	+ 58 +	1
+ + N	88 + +	88 58 +	+ 58 N	1
			Total	11

REMARKS:

Germination--91%.

SYMBOLS:

N = Non-conidial.
58 = 5801 (morphological).
88 = 8839 (leucine).
+ = Wild type allele of mutant in corresponding column.
A and a = Mating types.

8839, 5801, "N" A X 25 a
(continued)

SEGREGATION:

8839	1st Division	6
	2nd Division	5
5801	1st Division	11
	2nd Division	0
"N"	1st Division	5
	2nd Division	6

RECOMBINATIONS on basis of possible linkage:

8839-5801	Non-crossovers	2
	Single Crossovers	5
	Double Crossovers	4
8839-"N"	Non-crossovers	2
	Single Crossovers	8
	Double Crossovers	1
5801-"N"	Non-crossovers	3
	Single Crossovers	7
	Double Crossovers	1

aur a X 37501 A

SPORE PAIRS				NO. ASCI
ONE	TWO	THREE	FOUR	
aur a 37	aur a 37	+ A +	+ A +	1
+ A 37	+ A 37	aur a +	aur a +	2
+ A +	+ A +	aur a 37	aur a 37	1
aur a 37	aur a +	+ A +	+ A 37	1
+ A 37	+ A +	aur a 37	aur a +	2
+ A 37	+ A +	aur a +	aur a 37	1
+ A +	+ A 37	aur a +	aur a 37	1
aur A +	+ A +	aur a 37	+ a 37	1
+ a +	aur a +	+ A 37	aur A 37	1
aur a 37	+ a 37	+ A +	aur A +	1
aur a +	+ a +	+ A 37	aur A 37	1
aur A +	+ a 37	+ A +	aur a 37	1
aur A +	+ A 37	+ a +	aur a 37	1
aur A 37	+ A +	+ a +	aur a 37	1
+ a 37	aur A 37	aur a +	+ A +	1
+ A +	aur A 37	aur a 37	+ a +	1
			Total	18

REMARKS:

Germination--86%.

SYMBOLS:

aur = "aurescent".
37 = 37501 (leucine).
+ = Wild type allele of mutant in corresponding column.
A and a = Mating types.

aur a X 37501 A
(continued)

SEGREGATION:

37501	1st Division	9
	2nd Division	9
aur	1st Division	9
	2nd Division	9
"Sex"	1st Division	16
	2nd Division	2

RECOMBINATIONS on basis of possible linkage:

37501-aur	Non-crossovers	2
	Single Crossovers	12
	Double Crossovers	4
37501-Sex	Non-crossovers	3
	Single Crossovers	9
	Double Crossovers	6
aur-Sex	Non-crossovers	9
	Single Crossovers	9
	Double Crossovers	0

5801 a X 37501 A

SPORE PAIRS				NO. ASCI
ONE	TWO	THREE	FOUR	
58 +	58 +	+ 37	+ 37	2
58 37	58 37	+ +	+ +	1
+ +	+ 37	58 +	58 37	1
+ +	58 37	58 37	+ +	1
			Total	5

SEGREGATION:

5801	1st Division	4
	2nd Division	1
37501	1st Division	3
	2nd Division	2

RECOMBINATIONS on basis of possible linkage:

5801-37501	Non-crossovers	2
	Single Crossovers	1
	Double Crossovers	2

REMARKS:

Germination--90%.

SYMBOLS:

37 =37501 (leucine).
58 = 5801 (morphological).
+ = Wild type allele of mutant in corresponding column.
A and a = Mating types.

47313 a X "EMA"

SPORE PAIRS				NO. ASCI
ONE	TWO	THREE	FOUR	
47 a	47 a	+ A	+ A	3
+ A	+ A	47 a	47 a	2
			Total	5

SEGREGATION:

47313	1st Division	5
	2nd Division	0
"Sex"	1st Division	5
	2nd Division	0

RECOMBINATIONS on basis of possible linkage:

47313-Sex	Non-crossovers	5
	Single Crossovers	0
	Double Crossovers	0

REMARKS:

Isolated on minimal media.
Germination--80%.

SYMBOLS:

47 = 47313 (leucine).
+ = Wild type allele of mutant in corresponding column.
A and a = Mating types.
"EMA" = Emerson's A wild type.

33757, 47313 a X "EMA"

SPORE PAIRS				NO. ASCI
ONE	TWO	THREE	FOUR	
33 47 a	33 47 a	+ + A	+ + A	1
+ + A	+ + A	33 47 a	33 47 a	2
+ 47 a	33 47 a	+ + A	33 + A	1
+ 47 a	33 47 a	33 + A	+ + A	1
33 + A	+ + A	+ 47 a	33 47 a	1
+ 47 a	+ + A	33 + A	33 47 a	1
			Total	7

SEGREGATION:

33757	1st Division	4
	2nd Division	3
47313	1st Division	6
	2nd Division	1
"Sex"	1st Division	6
	2nd Division	1

RECOMBINATIONS on basis of possible linkage:

33757-47313	Non-crossovers	3
	Single Crossovers	4
	Double Crossovers	0
33757-Sex	Non-crossovers	3
	Single Crossovers	4
	Double Crossovers	0
47313-Sex	Non-crossovers	7
	Single Crossovers	0
	Double Crossovers	0

REMARKS:

Separation of types explained in text.
Double mutant parent derived from 33757, 4637 A X 47313 a.
Germination--100%.

SYMBOLS:

47 = 47313 (leucine).
33 = 33757 (leucine).
+ = Wild type allele of mutant in corresponding column.
A and a = Mating types.
"EMA" = Emerson's A wild type.

47313 a X 37501 A

SPORE PAIRS				NO. ASCI
ONE	TWO	THREE	FOUR	
+ + A	+ + A	47 37 a	47 37 a	1
47 37 a	47 + a	+ 37 A	+ + A	2
+ 37 A	47 + a	47 37 a	+ + A	1
			Total	4

SEGREGATION:

47313	1st Division	3
	2nd Division	1
37501	1st Division	1
	2nd Division	3
"Sex"	1st Division	3
	2nd Division	1

RECOMBINATIONS on basis of possible linkage:

47313-37501	Non-crossovers	0
	Single Crossovers	3
	Double Crossovers	1
47313-Sex	Non-crossovers	4
	Single Crossovers	0
	Double Crossovers	0
37501-Sex	Non-crossovers	0
	Single Crossovers	3
	Double Crossovers	1

REMARKS:

Germination--90%.

SYMBOLS:

47 = 47313 (leucine).
37 = 37501 (leucine).
+ = Wild type allele of mutant in corresponding column.
A and a = Mating types.