MUTATIONS AFFECTING THE REGULATION OF PRODUCTION OF THE ENZYMES OF LEUCINE SYNTHESIS IN NEUROSPORA[1]

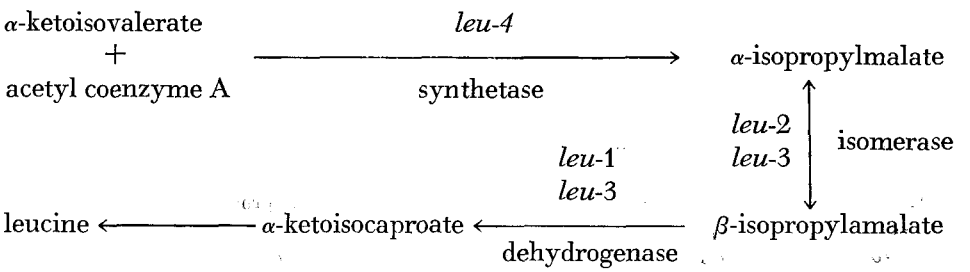
S. V. S. KASHMIRI[2] AND S. R. GROSS

Division of Genetics, Department of Biochemistry, Duke University, Durham, North Carolina 27706

Received July 28, 1969

AN analysis of enzyme production by wild type, feedback-insensitive and auxotrophic mutants suggested the involvement of at least two regulatory processes in the synthesis of the leucine biosynthetic enzymes of *Neurospora crassa*: 1) repression of synthesis of the first enzyme of the pathway as a function of intracellular leucine concentration and 2) the induced synthesis of subsequent enzymes of the pathway as a function of the concentration of the product of the first reaction, α-isopropylmalate (GROSS 1965). Such an interdependent regulatory mechanism should involve at least two different regulatory macromolecules and two or more genetic regulatory signal-receptor sites in controlling the function of the four genetically dispersed *leu* cistrons. One would expect, then, that regulatory mutants could be obtained that affect independently the repression and induction processes. In this paper we describe the isolation and characterization of several mutants in which the processes of repression and induction are altered differentially.

Enzymology and Genetics: Three enzymes are uniquely involved in leucine biosynthesis which proceeds in the following way:



The four *leu* cistrons are on three different linkage groups. Only *leu*-3 and *leu*-4 are linked and they are ten to fifteen units apart. The *leu*-4 cistron specifies the structure of the first enzyme of the pathway, α-isopropylmalate synthetase (the synthetase), which is sensitive to feedback inhibition by leucine (GROSS 1965; WEBSTER and GROSS 1965). The *leu*-2 and *leu*-1 cistrons specify the structure of

[1] Research was supported in part by National Science Foundation Grant GB-4489.

[2] Present address: Rockefeller University, New York, New York 10021.

isopropylmalate isomerase (the isomerase) and β-isopropylmalate dehydrogenase, respectively, while *leu-3* mutants produce only trace amounts of the isomerase and dehydrogenase. Because *leu-3* mutants complement *leu-2* mutants in heterokaryons only with difficulty, it was felt that the *leu-3* and the *leu2* cistrons might specify different polypeptide subunits of the isomerase (GROSS 1962). While no real evidence has been obtained to the contrary, it now seems more likely that the product of the *leu-3* cistron plays a regulatory role in the expression of *leu-2* and *leu-1* function (GROSS 1965).

MATERIALS AND METHODS

The three standard strains of Neurospora STD4A, STD7A, and STD8A as well as the fluoroleucine-resistant mutants used in this study are prototrophic inbred derivatives of *inos* 89601A.

Fluoroleucine-resistant mutants: Mutants designated as *FlR* or *flr* were derived from STD4A and selected on the basis of production of large colonies after growth, for two days at 34°C, on sorbose medium supplemented with 50 mg/l 5′,5′,5′-trifluoroleucine (fluoroleucine).

Three different mutagenic agents were used; ultraviolet light (UV), ^{60}Cobalt radiation, and N-methyl-N′-nitro-N-nitrosoguanidine (MNG). Usually a conidial suspension of optical density 1.0 at 550 mμ (all OD's listed were obtained at this wavelength) was exposed to a dose of radiation yielding 10 to 20% survival. The conidia were then diluted 100-fold and spread on fluoroleucine medium and incubated at 34°C for two days. When MNG was the mutagen, conidia were suspended in VOGEL's synthetic minimal medium N (VOGEL 1964) containing 1% sucrose and 5μg/ml MNG and incubated two hours at 34°C with aeration. The conidia were collected by filtration on Millipore filters, washed with synthetic medium and resuspended to yield an OD of 1.0, then spread at a dilution of 10^{-3} on fluoroleucine medium and incubated as above.

DK*I mutants: DK*I mutants were derived from MNG-treated STD4A conidia and were selected on the basis of their ability to grow in the presence of fluoroleucine at 25°C but not at 37°C even on complete medium in the absence of fluoroleucine.

Mutants of the DK*70 series*: Mutants of the *DK*70 series were derivatives of the leucine feedback-insensitive strain, *FlR* 70-2-63a. UV or MNG-treated conidia (about 4×10^4), were added to a liter of minimal sorbose-agar medium and the suspension layered in 5 ml aliquots over plates containing 20 ml minimal sorbose medium seeded with $2–4 \times 10^4$ conidia of a *leu*-1, *leu*-4 double mutant (D229-2-19A) and incubated at 34°C. After two to three days' growth at 34°C, halos appeared around all of the *FlR*70 colonies. Those colonies that produced halos significantly larger than the parental feedback-negative strain were isolated and purified by conidial reisolation.

Crosses: The sorbose plating method of NEWMEYER (1954) was used for random segregation analysis. The usual microdissection techniques were employed in isolating ordered tetrads and synthetic crossing medium (WESTERGAARD and MITCHELL 1947), appropriately supplemented, was used throughout.

Heterokaryosis: Heterokaryons were prepared by superimposing loopfuls of conidia from the two different strains on agar slants containing synthetic medium supplemented as required and incubated at 30°C. The nuclear ratios were determined by the procedure of PITTENGER (1964).

Growth determinations: Growth rates were determined both by the rate of linear progression (RYAN, BEADLE and TATUM 1943) and by change in mycelial mass. When the latter method was used, 20 ml of synthetic medium was inoculated with 0.05 ml of a conidial suspension of 0.15 OD and incubated at 34°C. Mycelia were collected by filtration, washed, dried for four hours at 70°C, equilibrated at room temperature, and weighed.

Preparation of extracts: Mycelia of the various strains were usually obtained after growth in one liter of synthetic medium, at 34°C with shaking for 24 to 72 hr depending upon the rate of growth of the strain. Growth was initiated with mycelia obtained from a 24 hr 20 ml

culture of the appropriate strain and mycelia were harvested after equivalent amounts of growth of the various mutants had been obtained.

Ammonium sulfate precipitates (0–75%) of mycelial extracts were prepared by the method of GROSS (1965) using 0.1 M potassium phosphate pH 6.5 buffer containing 2×10^{-4} M L-leucine to stabilize synthetase activity.

Enzyme assays: Enzyme assays were performed on the 0–75% ammonium sulfate precipitates after suspension in the same buffer used for harvesting and removal of particulate material by centrifugation for one hour at 100,000 $\times$ *g*. The synthetase activity was determined by the NEM (N-ethylmaleimide) assay procedure of WEBSTER and GROSS (1965). The isomerase was assayed by the method of GROSS, BURNS and UMBARGER (1963). The units of enzyme activity were those of GROSS (1965).

Assay for the secretion of leucine or a leucine analog: The mutant strains were grown for two days at 34°C. The mycelia were removed by filtration and 0.4 ml of the filtrate was applied to 2.3 cm Whatman 3M filter paper discs. After steam sterilization, the discs were applied onto solid synthetic *E. coli* medium (VOGEL 1964) seeded with a leucine auxotroph of *S. typhimurium*, *leu*-120. The appearance of a halo of bacterial microcolonies around a disc indicated the presence of either leucine or α-ketoisocaproate (the keto-analog of leucine), the only compounds found to effectively stimulate the growth of Salmonella leucine auxotrophs at pH 7.0 (JUNGWIRTH *et al.* 1961).

Amino acid uptake: Leucine uptake into a hot trichloracetic acid (TCA)-soluble fraction (the amino acid pool) and into a hot TCA-insoluble fraction (protein) was measured using mycelia obtained after 24 hr growth in 5 ml of synthetic medium containing 25 mg/l of L-leucine. Equal yields of mycelia from the two different strains used were obtained by using an inoculum of either 0.1 ml of a *leu*-1 or 0.3 ml of a *leu*-1,*DK*52 conidial suspension of 0.5 OD. The mycelia were collected by filtration on Millipore filters, washed with synthetic medium, then incubated in 2.0 ml of synthetic medium for 30 min. Uptake was initiated by the addition of 0.238 μg (1,814 $\mu\mu$ moles) of L-leucine-^{14}C (uniformly labeled, 275 μc/μ mole) and after incubation for an appropriate interval, the uptake was stopped by rapidly collecting the mycelia by filtration and washing them with six one ml aliquots of synthetic medium containing 10^{-4}M leucine (or lysine, when its uptake was measured) at 0°C. The mycelia were then extracted by boiling for five minutes in 5% TCA and the hot TCA extract collected by filtration. The extracted mycelia were washed three times with 1.0 ml of water and the washings added to the hot TCA extract. The extracted mycelia were then dried at 70°C, weighed and pulverized in counting vials. One ml aliquots of the hot TCA extracts were dried in counting vials, and 0.1 ml of water added before counting in scintillation fluid containing Triton X-100. Lysine uptake into the hot TCA soluble and insoluble fractions of *leu*-1 and *leu*-1,*DK*52 mycelia was measured in essentially the same way. The reaction mixture contained 150 mg/l of L-leucine and 0.66 μg (4,514 $\mu\mu$ moles) of L-lysine U^{14}C (221 μc/μ mole). Mycelia were obtained after 24 hr of growth in 5 ml of synthetic medium containing 150 mg/l of L-leucine but were not preincubated in the absence of leucine.

Miscellaneous: Spectrophotometric assays were performed at room temperature (25°–27°C). Protein was determined by the spectrophotometric method of WARBURG and CHRISTIAN (1942). 5′,5′,5′-trifluoroleucine was synthesized by the method of RENNERT and ANKER (1963) and β-isopropylmalic acid was a gift from Dr. J. CALVO.

RESULTS

Analyses of regulatory mechanisms of protein synthesis in Neurospora have been hampered by the scarcity of regulatory mutants. As a consequence, it was decided to try several of the methods of selection that have been successful in bacteria (MAAS 1961; CALVO and UMBARGER 1964). The first of the selection

methods tried involved screening for mutants resistant to trifluoroleucine, a compound which has been found to be an effective selective agent for leucine pathway specific regulatory mutants in *S. typhimurium* (CALVO and UMBARGER 1964). Conidia of strain STD4A, treated with either UV, ^{60}Co or MNG were plated on medium containing trifluoroleucine. Large resistant colonies appeared on plates containing many small sensitive colonies after two to three days at 34°C. The resistant colonies were purified by conidial reisolation, then reisolated from a cross to STD7a. Among 1062 resistant strains examined, 523 were sufficiently resistant to fluoroleucine to permit further study.

Relaxation of control of leucine biosynthesis should result in the overproduction of leucine or a precursor during growth. Therefore, 285 of the fluoroleucine-resistant mutants were assayed for the secretion of compounds that stimulate growth of *leu*-120, a leucine auxotroph of *S. typhimurium*, and 91, or 32% were found to be secretors. All of the secretors appeared to have mutated at a locus near mating type. An analysis of the enzyme complement of ten of the secretors revealed that all produced less synthetase and significantly more isomerase than wild type. More to the point, the synthetase produced by each of the secretors was found to be insensitive to feedback inhibition. Hence, all of the secretors were genotypically and phenotypically similar to *FlR*70 and *FlR*92, strains that produce a feedback-insensitive synthetase by virtue of a mutation in *leu*-4, the structural gene for the synthetase.

The enzymatic composition of 35 of the nonsecretors was examined. Except for *flr* 203, all were found to be normal. As indicated in Table 1, *flr* 203 produces somewhat more isomerase than normal when grown at 34°C without added leucine and synthesizes about five times more isomerase than the wild type when grown with leucine. Synthetase production by the mutant is somewhat less than normal and the enzyme is sensitive to leucine inhibition.

Genetic analyses of 52 nonsecreting fluoroleucine-resistant mutants revealed that 27 of them were either closely linked to, or alleles of *flr* 3. The *flr* 3 mutation is allelic with, or closely linked to the *mtr* locus on linkage group IV (no recombinants out of 1,000 segregants tested). Mutants at the *mtr* locus are resistant to inhibition by 4-methyltryptophan, *p*-fluorophenylalanine and ethionine as well

TABLE 1

Specific activity† of the synthetase and isomerase produced by flr *203 and* DK*I 342*

		Growth Medium			
		No supplement		300 mg leucine/l	
Strain	Mutagen	Synthetase	Isomerase	Synthetase	Isomerase
*flr*203-2-14A	^{60}Co	12.6 ± 3.5	132 ± 12 (6)	7.3 ± 2.1	84 ± 4 (3)
*DK*I 342-1-203A*	MNG	13.5 ± 4.7	256 ± 34 (4)	6.9 ± 2	264 ± 3.8 (3)
STD8A, wild type		24.2 ± 2.7	74 ± 2.7 (10)	7.1 ± 1.8	14.7 ± 5(2)

* Grown at room temperature 25–27°C. All other strains were grown at 34°C. Isomerase production by the wild type is only slightly higher at room temperature than at 34°C.

† ± Standard error. The number of assays is indicated in parentheses.

as to trifluoroleucine. The *mtr* locus has been shown to be involved in the production of a permease specific for hydrophobic amino acids (STADLER 1966). Interestingly, nine of the *mtr*-linked *flr* mutants are sensitive to inhibition by 4-methyltryptophan. Hence, if a single permease is involved, its specificity towards the individual amino acids may be subject to independent modification, and the *flr* 3-like mutants should provide useful material for the study of the permease.

Fluoroleucine-resistant, temperature-sensitive mutants were selected by plating MNG-treated wild-type conidia on medium containing fluoroleucine at 25°C. The fluoroleucine-resistant mutants obtained were then checked for temperature-sensitivity by transferring them to fluoroleucine-free synthetic medium at 37°C. Out of 742 fluoroleucine-resistant isolates tested, one mutant, *DK*I 342 failed to grow at 37°C even on complete medium. This strain was found to produce considerably more of the isomerase than wild type while producing a near-normal level of a leucine-sensitive synthetase (Table 1). Isomerase production by *DK*I 342 remains high even when grown in the presence of leucine. J. POLACCO (personal communication) has shown that *DK*I 342 is a double mutant and that relaxation of regulation of isomerase production is independent of the mutation that yields temperature sensitivity.

*DK*I 342 and *flr* 203 were the only presumptive regulatory mutants recovered by conventional selective procedures and in both, an alteration was observed only in the production of the isomerase and not in the synthetase, the first enzyme of the pathway. Clearly if synthetase regulatory mutants were to be found, some direct screening procedure for synthetase overproduction was required. The only simple criterion available for detecting the relaxation of regulation is the overproduction of leucine during growth. However, GROSS (1965) concluded that feedback inhibition is the major regulator of leucine production in Neurospora. This conclusion is supported further by the observation that all of the fluoroleucine-resistant mutants that secrete leucine produce a synthetase that is relatively insensitive to leucine inhibition. Some procedure, then, has to be devised to eliminate the obscuring effect of feedback inhibition on the relation of enzyme concentration to leucine production.

As a consequence, a method of screening directly for leucine oversecretors was devised using as the starting material, strain *FlR*70–2–63a, the most effective leucine-secreting, feedback-insensitive strain available. UV or MNG-treated conidia of the feedback-negative strain were plated on a lawn seeded with conidia of a *leu*-1,*leu*-4 double auxotroph (D229–3–19A). After incubation for three days at 34°C each of the *FlR*70 colonies was surrounded by a halo that resulted from cross-feeding of the double auxotroph. The halos were generally of uniform size but occasionally a larger, more intense halo was observed.

Out of about 100,000 colonies screened this way, 151 oversecretors were obtained and the enzyme levels in each determined. Eight of the isolates differed significantly from the parental strain. Once again, as indicated in Table 2, they were found to produce significantly more isomerase than the original feedback-insensitive strain without a detectable increase in the level of synthetase production. However, synthetase production by the original feedback-insensitive strain

TABLE 2

Specific activity† of the synthetase and isomerase produced by leucine oversecretors

Strain	Mutagen	Synthetase	Isomerase
*DK*70, 52a	UV	< 5	433 ± 32 (4)
*DK*70, 42a	MNG	< 5	309 ± 21 (2)
*DK*70, 17a	UV	< 5	361 ± 21 (2)
*DK*70, 28a	MNG	< 5	336 ± 15 (2)
*DK*70, 68a	MNG	< 5	317 ± 6 (2)
*DK*70, 108a	MNG	< 5	310 ± 5 (2)
*DK*70, 110a	MNG	< 5	312 ± 4 (2)
*DK*70, 133a	MNG	< 5	363 ± 7 (2)
*FlR*70–2–63a*	UV	< 5	206 ± 5 (2)

* The original feedback-insensitive strain.
† ± Standard error. The number of assays is indicated in parentheses.

is extensively repressed, and the level of activity in extracts is too low for accurate measurement. It was necessary, then, to determine whether or not the comparatively high levels of isomerase activity in the oversecretors resulted from cryptically high levels of synthetase production.

Two of the oversecretors, *DK*70,52 and *DK*70,42 were crossed to wild type and synthetase and isomerase production levels were determined in each of the phenotypically different segregants. Both crosses yielded, in addition to *FlR*70 and wild type, segregants that, like the parental strain, produced small fuzzy colonies on plates that conidiated with a characteristically sparse pattern when transferred to slants. These morphologically distinct segregants were of two types—one, like the parental oversecreting strain, containing a small amount of a feedback-insensitive synthetase and a high level of isomerase activity, and another, producing more of a feedback-sensitive synthetase than the wild type while producing near-normal amounts of the isomerase.

Enzyme production by three high-synthetase isolates, two obtained from *DK*70,52 (*DK*52–8–265A and *DK*52–8–219a) and one from *DK*70,42 (*DK*41–1–99A) are noted in Table 3. While neither *DK*42 nor *DK*52 produces anywhere

TABLE 3

The synthetase and isomerase activity of high synthetase segregants from crosses of DK*70, 42 and* DK*70, 52 by wild type*

	Growth Medium			
	No Supplement		300 mg leucine/l	
Strain	Synthetase	Isomerase	Synthetase	Isomerase
*DK*42–1–99A	43.5 ± 3.7	82.6 ± 8.9 (3)	24.3 ± 1.3	11.0 ± 2.1 (2)
*DK*52–8–265A	55.0 ± 8.2	53.9 ± 10.1 (13)	51.8 ± 7.5	19.1 ± 5.5 (8)
*DK*52–8–2–9a	37.4 ± 5.6	51.2 ± 8.1 (7)		
STD8A (wild type)	24.2 ± 2.7	74.0 ± 11.3 (10)	7.1 ± 1.8	14.7 ± 2.5 (2)
STD7a (wild type)	12.8 ± 4.3	68.0 ± 9.2 (14)		

near the levels of synthetase obtainable under conditions of maximal derepression (see values obtained for a *leu*-1 mutant grown on limiting leucine, Table 5), synthetase production by *DK*42 is repressed extensively by exogenously supplied leucine while synthetase production by *DK*52 is unaffected by exogenous leucine. Isomerase production levels of the mutant and the wild-type strains are approximately the same and exogenously supplied leucine reduces isomerase production to about the same level in mutant and wild-type strains.

*DK*52 seemed especially promising as a regulatory mutant because of its relative insensitivity to repression. A consistently puzzling observation, however, interfered with early attempts to analyze crosses involving *DK*52 on the basis of the enzyme complement of segregants. As indicated in Table 3, *DK*52 isolates of *a* mating type produce less synthetase than isolates of *A* mating type. A similar disparity in synthetase production was also observed for the *a* and *A* wild-type strains. Since the *leu*-4 locus (which specifies the structure of the synthetase) is about five units from the mating type locus (WEBSTER and GROSS 1965) it seemed likely that the *a* and *A* strains used either possessed slightly different *leu*-4 genes or, alternatively, one or more genes linked to the mating type locus affected the regulation of the *leu*-4 region. To check the involvement of genetic heterogeneity in the mating type–*leu*-4 region, *DK*52–8–265A was crossed to R108–9–8a, a *leu*-4 mutant. Prototrophic *DK*52 segregants of both mating types were checked for enzyme complement. Since *leu*-4 is closely linked to sex, only a few *DK*52 segregants with *a* mating type were obtained; but as indicated in Table 4, they possessed about the same amount of synthetase as the *A* parent and segregants. The *a* prototrophic segregants must have arisen by recombination between the *leu*-4 gene and some closely linked genetic material that was originally present in the *A* parent. The levels of synthetase activity produced in the *a* segregants, then, must be a function of the resident *leu*-4 allele and/or some genetic material linked to it and are not a function of mating type.

The regulation of synthetase and isomerase production in prototrophic strains can be studied only over a limited range of enzyme-production levels. The biosynthesis of these enzymes can be varied over a wide range in leucine auxotrophs grown in medium containing different amounts of leucine (GROSS 1965). Hence appropriate multiple mutants involving *leu*-1, *leu*-2, and *leu*-3 in combination with *DK*52 were prepared and the enzyme-production levels of the double mu-

TABLE 4

Synthetase levels of prototrophic DK*52 isolates from a cross R108–9–8a* × DK*52–8–265A*

Isolate No.	Mating type	Synthetase
378	*a*	56.3
433	*a*	58.7
439	*a*	56.0
371	*A*	55.8
401	*A*	65.0
402	*A*	59.2

TABLE 5

Synthetase and isomerase production by multiple mutants

Strain	Genotype	Growth medium: 25 mg leucine/l Synthetase	25 mg leucine/l Isomerase	300 mg leucine/l Synthetase	300 mg leucine/l Isomerase
*DK*52-12-316A	*leu*-1, *DK*52	338±37	371±35 (3)	93±5.1	248±45 (4)
D221-1-27A	*leu*-1	366±84	481±3 (3)	15±5.9	110±29 (4)
*DK*52-14-207A	*leu*-2, *DK*52	280±10	0 (2)	104±21	0 (5)
R86-8-40A	*leu*-2	140±16	0 (3)	25±5.1	0 (3)
*DK*52-16-331A	*leu*-3, *DK*52	76±13	trace (4)	30±2.9	trace (4)
*DK*52-18-499A	*leu*-3, *pan*-1, *DK*52*	74	trace (1)	26.2	trace (1)
R156-9-40A	*leu*-3	77±6.7	trace (3)	30±5.9	trace (4)
*DK*52-8-265A	*DK*52	55±8.2	40±0.5 (4)	52±12.5	19±5.5 (8)
STD8A	wild type	24±2.7†	74±11.3 (10)	7±1.8	15±2.5 (2)

* Growth medium supplemented with 50 mg Ca pantothenate in addition to leucine.
† Grown on minimal medium instead of medium supplemented with leucine.

tants compared to that of the single mutants grown on different levels of leucine.

The results, summarized in Table 5, indicate that when the amount of leucine supplied during growth is low, the *leu*-1 and *leu*-1,*DK*52 mutant strains produce about equally high levels of the synthetase and the isomerase. However, when leucine is supplied in excess, synthetase production in the *leu*-1,*DK*52 double mutant is repressed but only to levels about six times higher than the *leu*-1 mutant. The isomerase levels of both the single and double mutants were very high when the exogenous leucine concentration was low but again, in the presence of a relatively high concentration of leucine, isomerase production by the double mutant was at least twice that of the *leu*-1 single mutant. The repression of synthetase production in *leu*-2 and *leu*-2,*DK*52 mutants grown on high and low concentrations of leucine followed essentially the same pattern displayed by the *leu*-1 mutants. The only difference was that the derepressed level of synthetase production in the *leu*-2 mutants used here, derivatives of R86, was not as high as that exhibited by the *leu*-1mutants.

The *leu*-3,*DK*52 double mutant behaved differently from the corresponding *leu*-1 and *leu*-2,*DK*52 double mutants. GROSS (1965) has shown that synthetase production by the *leu*-3 mutant could not be maximally derepressed. The data of Table 5 further indicate that the insertion of *DK*52 into a *leu*-3 background has little effect on synthetase production either at maximal or minimal levels of repression. Hence, the regulatory role of *leu*-3 synthetase production is epistatic to that of *DK*52.

The phenotypic interaction between *DK*52 and *DK*52$^+$ was determined in heterokaryons prepared between leucine auxotrophs bearing *DK*52 and *DK*52$^+$ and also between *DK*52 and *DK*52$^+$ in *leu*$^+$ background. In each case, heterokaryosis was assured by the incorporation of heterologous, complementary auxotrophic requirements and by supplying only leucine during growth. The number of *DK*52 nuclei in each of the heterokaryons exceeded that of the *DK*52$^+$ nuclei thus minimizing dilution of the *DK*52 phenotype. The heterokaryons and each

TABLE 6

Synthetase levels of DK52/DK52+ *heterokaryons*

Genotype	Growth medium supplement	Synthetase	Nuclear ratio *DK52/DK52+*
leu-2, *pan*-1, *DK*52	50mg pan + 300mg leu	110	
leu-4, *ad*-5, *DK*52+	20mg adenine + 25mg leu	0	
[*leu*-2, *pan*-1, *DK*52 / *leu*-4, *ad*-5, *DK*52+]	300mg leu	10.8	1.80:1
leu-1,2, *pan*-1, *DK*52	50mg pan + 300mg leu	60	
[*leu*-1,2, *pan*-1, *DK*52 / *leu*-4, *ad*-5, *DK*52+]	300mg leu	16.5	3.37:1
leu-1,2, *inos*, *DK*52+	15mg inos + 300mg leu	18.7	
[*leu*-1,2, *pan*-1, *DK*52 / *leu*-1,2, *inos*, *DK*52+]	300mg leu	20.0	1.95:1
pan-1, *DK*52	50mg pan + 300mg leu	46.3	
inos, *DK*52+	15mg inos + 300mg leu	6.6	
[*pan*-1, *DK*52 / *inos*, *DK*52+]	300mg leu	6.2	1.26:1

Heterokaryons are noted in brackets. Pan, inos and leu are calcium pantothenate, i-inositol and L-leucine. The amount of supplement is in mg/l.

of the auxotrophic components were grown under conditions of maximal repression. As reported in Table 6, synthetase production was low in each of the heterokaryons which indicates that *DK*52+ is dominant to *DK*52.

In order to determine whether failure to fully repress synthetase production in the *DK*52 mutant and the corresponding leucine auxotrophs bearing *DK*52 is due to an impairment in the ability to concentrate and/or retain leucine, the growth of the wild type as a function of leucine supplied was compared to that of the single mutants *DK*52 and *leu*-1 as well as to that of the *leu*-1,*DK*52 double mutant. As illustrated in Figure 1, growth of the *DK*52 prototrophic strain, like the wild type, was not stimulated by the addition of leucine. *DK*52, however, yielded only about half as much mycelia as the wild type at all leucine concentrations. For some unknown reason, the *leu*-1,*DK*52 double mutant yielded more mycelia at plateau than the *DK*52 single, but the maximum level of growth obtained by the double mutant was only a little more than half that of *leu*-1 single. The mycelial yield of a *leu*-2,*DK*52 double mutant as a function of leucine concentration was found to be essentially identical to that obtained with the *leu*-1,*DK*52 strain. The data of Figure 2A indicate that the rate of progression on solid medium (RYAN 1943) of wild-type mycelia is considerably faster than *DK*52 and that the growth rate of both strains is independent of the amount of leucine supplied. Supplementation with isoleucine, valine, lysine or methionine at concentrations as high as 75 to 150 mg/l did not stimulate growth of *DK*52. The growth progression data presented in Figure 2B indicate that the maximal rate of growth attainable by the *DK*52,*leu*-1 double mutant at any leucine concentration is less than half that of the *leu*-1 single mutant.

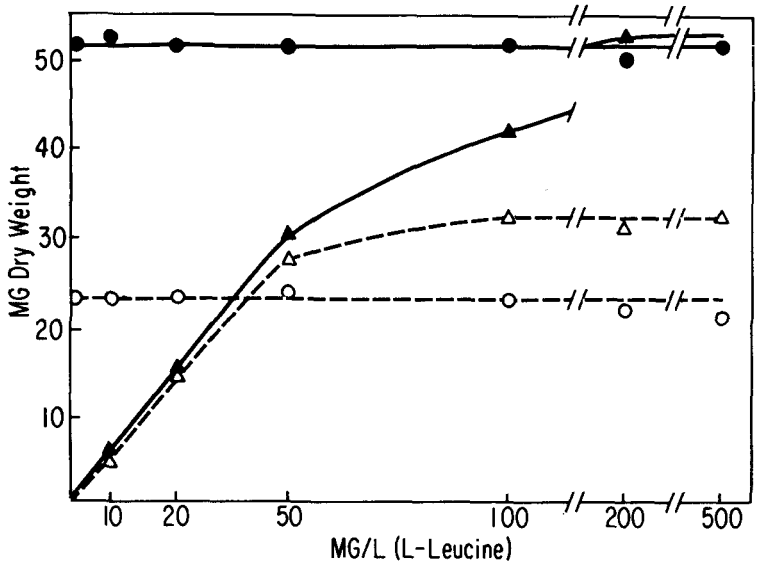


FIGURE 1.—Total yields of mycelia as a function of L-leucine concentration after growth in liquid medium for 73 hours at 34°C. Wild type (●), *DK*52 (○), *leu*-1 (▲) and *leu*-1,*DK*52 (△). The points represent the averages of two determinations.

Failure of exogenously supplied leucine to stimulate the growth of *DK*52 coupled with the observation that the level of isomerase production by the mutant is normal despite the production of two to three times more than the normal amount of synthetase suggests (1) that enough leucine is produced by *DK*52 to support its growth at the maximal attainable rate and (2) that the intracellular

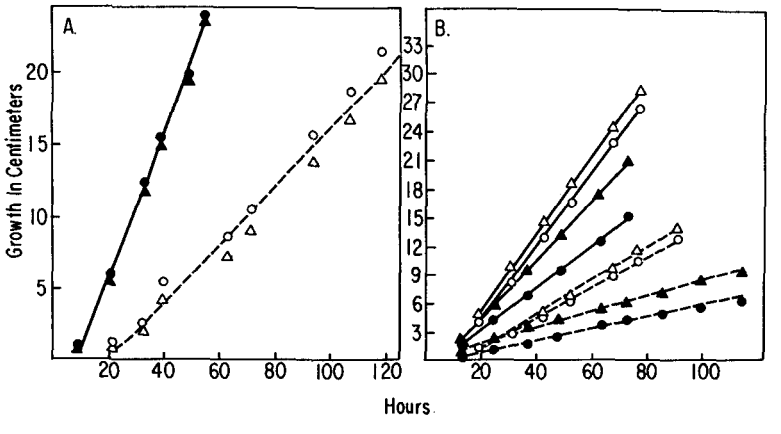


FIGURE 2.—The linear growth progression of *DK*52 and *leu*-1,*DK*52. (A). Growth of wild type (——) and *DK*52 (– – –) at 34°C on solid medium supplemented with 5 mg/l (circles) and 40 mg/l (triangles) L-leucine. (B). Growth of *leu*-1 (——) and *leu*-1,*DK*52 (– – –) at 34°C on solid medium supplemented with 5 mg (●), 10 mg (▲), 40 mg (○) and 160 mg (△) L-leucine/l.

concentration of leucine is high enough to feedback-inhibit the synthetase and restrict the production of α-IPM, the inducer of isomerase synthesis. Indeed, an auxonographic analysis of culture filtrates of *DK*52 revealed that a small amount of leucine or α-ketoisocaproate is secreted into the medium during growth. The conclusion, therefore, seems inescapable that the mutant produces more than enough leucine or leucine precursor for growth but cannot fully utilize intracellular leucine to repress the production of the synthetase to normal levels.

To check whether the impaired regulatory function is correlated with an impaired ability to incorporate leucine into protein or to concentrate leucine into some intracellular free amino acid pool, the incorporation of leucine was measured into the TCA soluble pool (presumably free leucine) and the TCA-insoluble fraction (protein) of *leu*-1,*DK*52, and *leu*-1 mycelia. The internal amino acid pool was depleted of leucine by growing both strains in medium containing a limiting amount of leucine followed by a half hour incubation in minimal medium at 34°C before adding leucine $U^{14}C$. The results illustrated in Figure 3A clearly indicate that the uptake of leucine into the amino acid pool of the *leu*-1,*DK*52 double mutant is only about one third that of *leu*-1 and incorporation into protein is reduced equivalently. The *DK*52 mutation, then, imposes some problems in concentrating leucine into an internal pool. However, the question raised is whether the low rate of uptake is an effect rather than a cause of the lower rate of protein synthesis.

If the leucine permeability is specifically affected by the *DK*52 mutation, the uptake into the amino acid pool of an amino acid structurally unrelated to leucine should be normal. Accordingly, the rate of lysine uptake by *leu*-1,*DK*52 was compared to that of *leu*-1. The intracellular concentration of lysine could not be depleted by any simple procedure and depletion of intracellular leucine should be irrelevant to lysine uptake. Therefore, both the double and single mutants were grown in excess leucine (150 mg/l) and lysine uptake was determined in leucine-containing medium without an intervening starvation period. The data in Figure 3B indicate that more lysine than leucine was incorporated into the amino acid pool. Nonetheless, *leu*-1,*DK*52 incorporated less than half the amount of lysine into the amino acid pool that was incorporated by the *leu*-1 mutant and the incorporation of lysine into protein by *leu*-1,*DK*52 was about one-eighth that of *leu*-1. Despite the fact that leucine and lysine uptake were measured under different conditions, the uptake into the free amino acid pool and the incorporation into protein of both amino acids were significantly lower in *leu*-1,*DK*52 than in *leu*-1. Hence, it seems unlikely that the primary effect of the *DK*52 mutation is on a leucine-specific permeability system.

The characteristic phenotype of *DK*52, slow growth and sparse conidiation as well as the formation of fuzzy colonies when plated on sorbose medium, allowed the identification of *DK*52 segregants from crosses with reasonable accuracy. Invariably, however, considerably less than 50% *DK*52 like progeny were recovered from crosses with markers in each of the seven linkage groups. Linkage was not detected to any markers other than those in the right arm of linkage group I. The data recorded in Table 7 suggest that *DK*52 might be loosely linked

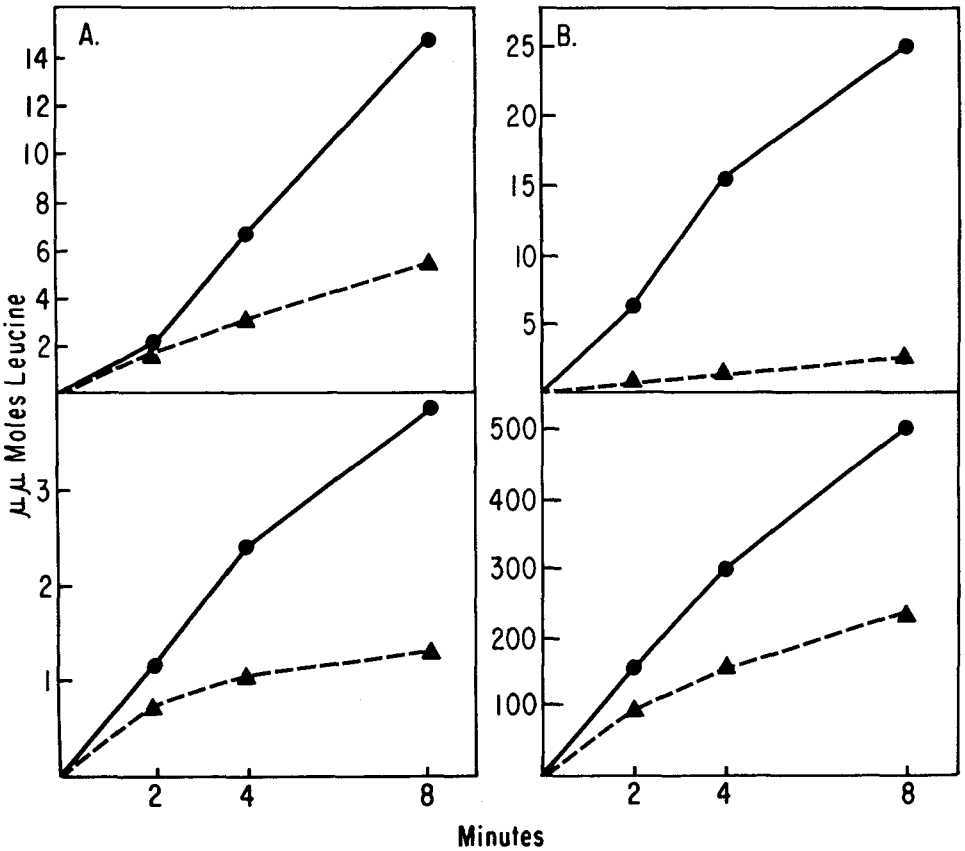


FIGURE 3.—Amino acid incorporation by *leu*-1,*DK*52. (A). The incorporation of L-leucine into TCA insoluble material (upper figure) and the TCA soluble pool (lower figure). ●, Leucine incorporation by *leu*-1; ▲, by *leu*-1,*DK*52. (B). The incorporation of L-lysine into TCA insoluble material (upper figure) and the TCA soluble pool (lower figure). ●, lysine incorporation by *leu*-1; ▲, by *leu*-1,*DK*52.

to *al*-2 and *nic*-1. However, the recovery of only half, or less, of the expected number of *DK*52 segregants, and apparent selection against specific combinations cast doubt on the reliability of such an analysis. Crosses of *DK*52 to markers more proximal to *al*-2 on linkage group I failed to indicate any linkage and crosses involving markers such as *osmotic* and *flame* more distal to *nic*-1 were difficult to analyze because of the recovery of a large number of morphologically unidentifiable progeny and poor *DK*52 recovery.

The failure to detect reasonably close linkage of *DK*52 to any of some 20 loci surveyed suggested that the *DK*52 phenotype may result from a complicated genetic interaction involving two or more genes. Therefore, the segregation of *DK*52 was determined in ordered tetrads obtained from crosses of *DK*52 by wild type, *DK*52 by *pyr*-4 and *al*-2,*DK*52 by wild type. The pertinent tetrad data obtained from the three crosses are summarized in Table 8. Out of 83 asci dissected,

TABLE 7

Two-point crosses involving DK*52,* al-*2 and* nic-*1*

Cross	Segregants	Recombination frequency	Frequency of *DK*52 among progeny
15300a × *DK*52-8-265A (*al*-2) (52)	Parental +, *al* 261 *52*, + 58	0.39	0.17
	Recombinant +, + 170 *52*, *al* 32		
3416a × *DK*52-8-265A (*nic*-1) (52)	Parental + *nic* 278 *52* + 143	0.37	0.27
	Recombinant + + 209 *52 nic* 35		

49 yielded representatives of all four spore pairs and in 32 of the 49 tetrads analyzed only one pair of the four was phenotypically *DK*52. Furthermore, in most 1:3 tetrads one of the non-*DK*52 spore pairs was distinguishable from the others because of slightly poorer conidiation and pigment formation. A 1:3 segregation of this sort indicates the involvement of at least two genes.

The hypothesis then is that the *DK*52 phenotype results from an interaction of two mutant genes, x and y, in a common genome. Strains of genotype x^+y, xy^+, or x^+y^+ are normal with regard to the regulation of synthetase production. Furthermore, either the xy^+ or x^+y segregant has a slightly different morphology from that of wild type and *DK*52. This hypothesis accounts for the failure to recover significantly more than 25% *DK*52 segregants from most crosses and for the appearance of unscorable morphological variants. The segregation of mating type and *al*-2 from *DK*52 is summarized in Table 9 and suggests that

TABLE 8

The segregation of the DK*52 phenotype in tetrads*

Cross	Tetrads isolated	Segregation of *DK*52 2:2	1:3	0:4
*DK*52-8-265A × 36601a (52) (*pyr*-4)	4	0	4	0
*DK*52-8-265A × STD7a (52) (wild type)	5	1	3	1
*DK*52-15-95a × STD8A (52 *al*-2) (wild type)	40	8	25	7
Total	49	9	32	8

TABLE 9

Summary of the segregation of DK52, *mating type and* al-2 *in tetrads*

Cross	Mating type		*al*-2	
	Parental (52A)	Recombinant (52a)	Parental (52 *al*)	Recombinant (52 *al*+)
*DK*52-8-265A × 36601a (52) (*pyr*-4)	1	3	..	..
*DK*52-8-265A × STD7a (52) (wild type)	3	2	..	..
*DK*52-15-95a × STD8A (52 *al*-2) (wild type)	22	19	27	14
Total	26	24	27	14

either the x or y of *DK*52 is loosely linked to *al*-2. Linkage to the mating type locus was not detectable.

The levels of synthetase activity in a member of each spore pair of three different asci are listed in Table 10. As is evident, a high level of synthetase activity is uniquely associated with the *DK*52 phenotype. Clearly then, if the notion of the involvement of two genes in the determination of the *DK*52 phenotype were correct, an ascus displaying 1:3 segregation should have one spore pair of each of x^+y, xy^+ and x^+y^+ in addition to the xy pair that yields the *DK*52 enzymology and morphology. Hence crossing members of each of the non-*DK*52 pairs to each other should yield one combination that can generate *DK*52-like segregants. Cultures derived from spore pairs 1 and 2 of tetrad VI of Table 10 were crossed to spore pair 4. Segregants phenotypically identical to *DK*52 were obtained only from the cross of spore pair 2 by 4 and not from 1 by 4. The

TABLE 10

The synthetase production level of tetrad segregants

Cross	Ascus number	Ascospore pair	Phenotype	Mating type	Synthetase
		1	+	*a*	10.3
		2	+	*a*	8.9
	VI	3	52	*A*	65.7
		4	+	*A*	24.5
*DK*52-8-265A × STD7a (52) (wild type)					
		1	52	*A*	51.3
		2	+	*A*	29.0
	X	3	+	*a*	10.1
		4	+	*a*	7.6
*DK*52-15-95a × STD8A (52 *al*-2) (wild type)					
		1	+ al	*A*	19.7
		2	+ +	*A*	20.2
	III	3	+ +	*a*	10.1
		4	+ al	*a*	15.3

TABLE 11

Genetic analysis of DK*42*

Zygote genotype and recombination frequency					Segregants							
					Parentals				Single exchanges 2			
					+	*tryp*	+	77	+	*tryp*	*42*	24
					ylo	+	*42*	89	*ylo*	+	+	28
+	**1**	*tryp*-2	**2**	+								
ylo	0.19	+	0.25	*DK42*								
					Single exchanges **1**				Double exchanges **1** & **2**			
					+	+	*42*	14	+	+	+	10
					ylo	*tryp*	+	22	*ylo*	*tryp*	*42*	6

frequency of recovery of *DK*52-like segregants was about 20%, and only those segregants that were morphologically like *DK*52 produced two to three times more than the normal amount of enzyme.

A genetic analysis of *DK*42 indicated that it is clearly different from *DK*52. The data in Table 11 obtained from a cross of a *ylo,DK*42 double mutant to *tryp*-2 indicate that *DK*42 is a mutation in the right arm of linkage group VI distal to *tryp*-2.

The position of the *flr* 203 locus has not been determined with any precision because of scoring ambiguity. The data available, however, suggest that it is in the left arm of linkage group I. Crosses involving the other mutation that leads to isomerase overproduction, *DK*I 342 and derivatives thereof, have been too infertile for genetic analysis.

DISCUSSION

The report here of the recovery of two classes of mutants, one that specifically produces elevated levels of the synthetase and another in which only the level of isomerase production is higher than normal lends further support to the proposal (GROSS 1965) that a dual regulatory mechanism is involved in the control of the synthesis of the leucine biosynthetic enzymes of Neurospora. The data obtained do not permit determining whether the phenotypes studied result from primary effects on those regulatory molecules directly responsible for determining the rate of transcription and/or translation of the four *leu* cistrons. However, they do suggest the involvement of at least two regulatory elements, each affected by mutation independently of the other.

The two mutants affecting synthetase production, *DK*42 and *DK*52, differ markedly from each other genetically as well as physiologically. Synthetase production by *DK*52 is higher and more refractory to repression by leucine than is synthetase production by *DK*42. Isomerase production by both mutants grown without leucine is essentially the same as that of wild type and is reduced to an almost negligible amount when leucine is supplied. Since it has been shown (GROSS 1965) that there is a direct relation between the amount of the inducer, α-isopropylmalate, and the level of isomerase production, both mutants must be

able to produce, retain, and concentrate exogenously supplied leucine in order to regulate effectively α-isopropylmalate synthesis by feedback inhibition. It would seem then, that in both mutants, leucine functions less effectively as a co-repressor than as a feedback inhibitor. This may indicate some difficulty in the formation of a true co-repressor from leucine or some secondary effect on co-repressor production.

The enzyme production levels in the *leu*-3, *DK* 52 double mutant indicate that the *leu*-3 mutation is epistatic to *DK*52 and, as a consequence, probably acts at some point in the regulatory processes different from *DK*52. The requirement for the presence of a *leu*-3$^+$ gene for the production of active isomerase and dehydrogenase, as well as its requirement for complete derepression of synthetase production has been reported previously (GROSS 1965). It was suggested then, that in addition to providing information relevant to the structure of the isomerase and dehydrogenase, the *leu*-3$^+$ gene might play a role in coordinating the synthesis of all three leucine biosynthetic enzymes. It is now necessary to consider that the *leu*-3$^+$ product may act as a "positive" controlling element necessary for derepression of the synthetase as well as the isomerase and dehydrogenase (GROSS 1969).

Unlike *DK*42, which involves a mutation in linkage group VI distal to *tryp*-2, the genetics of *DK*52 is somewhat complicated. Tetrad analysis revealed that *DK*52 contains two mutated genes neither of which has a significant effect on the regulatory mechanism by itself. Furthermore, the other characteristics of *DK*52—relatively slow growth and the pattern of conidia production—appear only when both mutated genes are present. The linkage data obtained suggest that one of the genes may be on linkage group I, linked loosely to *al*-2. Since neither of the single mutants extracted from *DK*52 resembles *DK*42 phenotypically or genetically, it must be assumed for the present, at least, that three separate loci have been identified and that the three are involved in the regulation of synthetase production.

The mutants that display altered levels of isomerase production, *flr*203 and *DK*I 342, have been studied only cursorily. Both produce higher levels of isomerase activity than wild type under all conditions tested but relaxation of isomerase regulation can be most easily ascertained when synthetase production is extensively repressed by leucine. Since production of the inducer, α-isopropylmalate, is a function of the amount of synthetase present as well as the effectiveness of feedback inhibition (GROSS 1965), either feedback inhibition does not function efficiently in these mutants, or the mutations are indeed "regulatory" in the sense that they affect the rate of eventual translation of the isomerase structural gene(s). A defect in leucine retention or permeability does not explain the high isomerase production levels in *flr*203 and *DK*I 342 for the following reasons: (1) The mutants synthesize enough leucine and can concentrate a sufficient amount of exogenously supplied leucine to repress synthetase production. (2) The response of the isomerase regulatory mechanism of the mutant strains to exogenously supplied leucine differs markedly from that of a known permeability mutant, flr_3, in which isomerase production is reduced to normal levels by leucine.

The regulatory mutants obtained and the difficulty encountered in obtaining them point to the probability that the mechanism governing the synthesis of the leucine biosynthetic enzymes may be rigorously controlled by several interacting regulatory mechanisms in Neurospora. Surprisingly, no operator mutants or extreme regulator-deficient mutants have been obtained. Only feedback-insensitive and permease-deficient mutants were recovered in Neurospora at frequencies near that expected of bacterial systems.

SUMMARY

Mutants of Neurospora affecting the regulation of synthesis of the leucine biosynthetic enzymes were obtained through the use of the conventional selection methods of analog resistance and temperature sensitivity as well as a specially designed method of selecting for leucine oversecretors starting with a strain that produces a leucine-insensitive α-isopropylmalate synthetase. Two classes of mutants were obtained; one that overproduced the synthetase while producing normal levels of the isomerase, and another that produced normal levels of the synthetase but overproduced the isomerase. The two mutants that produced more than the normal amount of synthetase proved to be genetically and physiologically different from each other. Synthetase production by *DK*42 is somewhat sensitive to leucine-mediated repression while *DK*52 is more markedly refractory to repression by leucine. The *DK*42 phenotype results from a mutation on linkage group VI distal to *tryp*-2. The genetics of *DK*52 is more complicated and, on the basis of tetrad analysis, apparently involves two mutant genes one of which may be linked to *al*-2 on linkage group I. The data obtained suggest that the effect on overproduction of the synthetase by *DK*52 is not a result of a specific defect in leucine uptake, retention, or synthesis. The *DK*52 phenotype is recessive in heterokaryons and the limiting effect of *leu*-3 mutations on the derepression of the synthetase is epistatic to *DK*52. Mutants that overproduce the isomerase, *flr* 203 and *DK*I 342, do so under conditions that lead to extensive repression and feedback inhibition of the synthetase. The data obtained provide further support to the notion of the involvement of a system of multiple interacting regulatory elements that govern the rate of transcription and translation of the genetically dispersed structural genes for the enzymes of leucine biosynthesis in Neurospora.

LITERATURE CITED

CALVO, J. and H. UMBARGER, 1964 Mutants of *Salmonella typhimurium* constitutive for leucine biosynthetic enzymes. Fed. Proc. (Abstract) **23**: 377.

GROSS, S. R., 1962 On the mechanism of complementation at the *leu*-2 locus of Neurospora. Proc. Natl. Acad. Sci. U.S. **48**: 922–930. ——, 1965 The regulation of synthesis of leucine biosynthetic enzymes in Neurospora. Proc. Natl. Acad. Sci. U.S. **54**: 1538–1546. ——, 1969 Genetic regulatory mechanisms in the fungi. Ann. Rev. Genetics **3**: 395–424.

GROSS, S. R., R. O. BURNS and H. E. UMBARGER, 1963 The biosynthesis of leucine. II. The enzymic isomerization of β-carboxy-β-hydroxyisocaproate and α-hydroxy-β-carboxyisocaproate. Biochemistry **2**: 1046–1052.

JUNGWIRTH, C., P. MARGOLIN, E. UMBARGER and S. R. GROSS, 1961 The initial step in leucine biosynthesis. Biochem. Biophys. Res. Commun. **5**: 435–438.

MAAS, W. K., 1961 Studies on repression of arginine biosynthesis in *Escherichia coli*. Cold Spring Harbor Symp. Quant. Biol. **26**: 183–191.

NEWMEYER, D., 1954 A plating method for genetic analysis in Neurospora. Genetics **39**: 604–618.

PITTENGER, T. H., 1964 Crossing techniques for large numbers of isolates. Neurospora Newsletter **6**: 23.

RENNERT, O. M. and H. S. ANKER, 1963 On the incorporation of 5′,5′,5′-trifluoroleucine into proteins of *E. coli*. Biochemistry **2**: 471–476.

RYAN, F. J., G. W. BEADLE and E. L. TATUM, 1943 The tube method of measuring the growth rate of Neurospora. Am. J. Botany **30**: 784–799.

STADLER, D. R., 1966 Genetic control of the uptake of amino acids in Neurospora. Genetics **54**: 677–685.

VOGEL, H. J., 1964 Distribution of lysine pathways among fungi: evolutionary implications. Am. Naturalist **98**: 435–446.

WARBURG, O. and W. CHRISTIAN, 1942 Isolierung und Kristallisation des Gärungsfermentes Enolase. Biochem. Z. **319**: 384–421.

WEBSTER, R. E. and S. R. GROSS, 1965 The α-isopropylmalate synthetase function. Biochemistry **4**: 2309–2318.

WESTERGAARD, M. and H. K. MITCHELL, 1947 Neurospora. V. A synthetic medium favoring sexual reproduction. Am. J. Botany **34**: 573–577.