A SERIES OF HISTIDINELESS MUTANTS OF NEUROSPORA CRASSA

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LEIN, MITCHELL and HOULAHAN (1948) have reported the isolation of a histidine-requiring mutant of Neurospora by means of a variation in the technique described by BEADLE and TATUM (1945). They also found evidence that histidineless mutants had not previously been obtained because they were inhibited by substances present in the various media used for their isolation.

Since this time, six other histidineless mutants have been isolated in this laboratory using the technique described by LEIN *et al.* The present report is concerned with investigations on the biochemistry and genetics of these seven histidineless Neurospora mutants that have now been obtained. Isolation numbers of the mutants are C84, C85, C91, C94, C140, C141 and T1710. The seven mutants represent at least four different genetic loci as will be demonstrated. All are inhibited in the same manner as the strain reported previously.

EXPERIMENTAL

Histidine requirement

Stock cultures of the histidineless mutants are kept on minimal medium (RYAN, BEADLE and TATUM, 1943) supplemented with L-histidine since the mutants fail to grow on media containing hydrolyzed casein, yeast extract, liver extract, autolysate of Neurospora mycelium or corn meal infusion. (C140, C141, and T1710 were tested only on media containing hydrolyzed casein, yeast extract or autolysate of Neurospora mycelium. They will not grow on these media.)

The quantity of L-histidine hydrochloride monohydrate required for half-maximum growth by C84, C91, and C94 is 0.5 mg in 20 ml of medium. C85 and C140 require only about half this amount, while C141 requires slightly more (about 0.6 mg in 20 ml medium). T1710 needs only 0.09 mg for half-maximum growth. All of the mutants except C141 give straight-line growth curves which indicate a regular increase in growth with an increase in the amount of histidine supplied up to the point of maximum growth. C141, however, gives an S-shaped curve and shows no appreciable growth when less than 0.2 mg of histidine in 20 ml medium is furnished. Histidine requirements

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were determined by measuring the dry weights of mycelia obtained from cultures grown for 4 days at 25°C in 125-ml Erlenmeyer flasks containing 20 ml of basal medium supplemented with varying amounts of L-histidine HCl.

Other compounds have been tested for growth-stimulating properties on C84 and C94. Of those tested none has been found to promote growth of these mutants. Those compounds found inactive are imidazole, imidazole acrylic acid, imidazole lactic acid, histamine, histidinol, imidazole glyoxylic acid, methyl imidazole, imidazole carboxylic acid, imidazole aldehyde, 4-amino-5-imidazole carboxamide, 4-amino-5-imidazol carboxamidine, imidazole methanol, thiol-histidine, 1-methyl histidine, adenine, cytidine, DL-alanine, glycine, DL-serine, L-proline, L-oxyproline, L-aspartic acid, L-glutamic acid, guanidine and glycocyamine, as well as the amino acids DL-leucine, L-isoleucine, DL-valine, DL-phenylalanine, L-tryptophan, L-lysine, L-arginine, DL-methionine and DL-threonine. Roughly half of these amino acids are slightly stimulatory in the presence of histidine.

Inhibition

As investigations of biochemical mutants proceed it becomes increasingly obvious that acquisition by mutation, of a new growth requirement is often accompanied by other changes in the physiological behavior of the organism. One such change leads to inhibition of the mutant by normal metabolites in concentrations which do not affect the growth of the wild type (DOERMANN 1946) (TEAS, HOROWITZ and FLING 1949). This is the case with this series of histidineless mutants.

It was found that 5 mg of " Vitamin Free " casein acid hydrolysate (Nutritional Biochemical Corp.) brought about complete inhibition in the presence of 0.5 mg of L-histidine HCl. The amino acids known to be in casein, when tested singly in 2-mg quantities with the above amount of histidine, produced no inhibition, but a mixture of these was inhibitory. By testing various combinations it was found that if either L-arginine or L-lysine is present, then any one of the following will be inhibitory: L-leucine, DL-isoleucine, DL-valine, DL-methionine, glycine, L-tyrosine, DL-phenylalanine, L-tryptophan or histamine. No inhibition by the following compounds has been observed: DL-serine, DL-threonine, L-proline, L-oxyproline, L-cystine, L-aspartic acid, L-glutamic acid, tyramine, ethylamine, alanine, imidazole and imidazole acrylic acid. The above compounds, both active and inactive, were tested in the presence of tyrosine to determine whether they could replace lysine or arginine. None of them did so, nor did DL-citrulline, guanidine or glycocyamin. DL-Ornithine and α-amino-ε-guanidino caproic acid, however, were found to be effective.

Extensive tests with mutant C94 have provided quantitative data on the inhibitory ratios of histidine, arginine and tyrosine. In order for complete inhibition to take place the molar concentration of tyrosine must exceed that of histidine, but if tyrosine is increased then the arginine concentrations may

be much lower than that of histidine. Arginine alone produces about half inhibition in a molar concentration which is 7 times the histidine concentration, but increasing arginine to make a 14-fold difference has no further effect. Inhibition by tyrosine alone has not been observed.

Inhibitory combinations other than tyrosine and arginine have been tested less thoroughly, but tests which have been made show that with tyrosine, either lysine or ornithine has about the same activity as arginine. The tests indicate no great difference in effectiveness of the compounds which replace tyrosine. Concentrations necessary for complete inhibition vary with the age of the culture from which the inoculations are made, higher concentrations being required if very young cultures (two or three days old) are used. Also the inhibition is not nearly so great when the mutants are grown on solid agar medium which contains the inhibitors.

Experiments designed to test whether the inhibitions of histidine mutants, described above, are due to the action of modifier genes suggest that this is the case but the genetic analysis is not yet complete.

Genetic analysis

Six of the histidineless mutants were found among 1760 selected mutant spores (LEIN *et al.* 1948) isolated from the crosses of wild-type 7A with wild-type 8a after irradiating one or the other with ultraviolet. All wild-type crosses do not seem to produce viable histidineless mutants. For example, 1633 selected single ascospore isolates from two crosses between Abbott 4A and 8a yielded no histidineless mutants.

Tests for formation of heterocaryons between the mutants were carried out (BEADLE and COONRADT 1944). Flasks containing 20 ml basal medium supplemented with 0.01 mg of histidine HCl per ml of medium were inoculated with each mutant separately and in all possible combinations of two. C85 and C91 were not included. The amount of histidine added permits germination of the conidia of the above strains but is insufficient for much growth. It was found that heterocaryons are formed in all cases except in combinations between C94, C140 and T1710 and between C94 and C84.

This experiment provided evidence that at least three different genetic loci are involved. This was first verified by counting random spore types from crosses of each mutant with every other mutant. Ascospores, when ripe, were collected on solid minimal medium in Petri plates, heat activated at 60°C for 30 minutes and then incubated overnight at 25°C. The plates were then examined for wild-type and histidineless spores. Mutant spores germinate on this medium but the hyphae do not grow whereas growth of the wild-type continues until the plate is covered. Since both members of each cross are histidineless mutants, any wild-type spore must be the result of a genetic recombination which eliminated both histidineless genes of the parents. These genes must, therefore, have different loci. Counts of wild-type and mutant spores from each cross yielded evidence that mutants C84, C94, C140 and C141 are genetically different. C84 behaves as an allele of C85 and C91, and

C140 as an allele of T1710. The latter two mutants, C140 and T1710 are linked to C94.

The results obtained from the above experiments were confirmed by dissecting asci from each of the crosses between histidineless mutants. All 8 spores from an ascus were removed in order and placed in a row on a Petri plate of solid minimal medium, 30 to 50 rows per plate. The spores were activated, allowed to germinate and grow overnight and then they were examined. Classes of asci recorded were those having eight histidineless spores, those having six histidineless and two wild, and those having four histidineless and four wild. Pertinent data obtained from these crosses are given in table 1. The results show that the mutant genes of C84 and C141 are not linked to each other or to C94, C140 or T1710. The last three do show linkage and

TABLE 1

Genetic analysis of histidineless mutants by dissection of asci progeny from intercrosses.

Cross	Ascus types*						Total No. asci	Linkage
	8H		6H–2+		4H–4+			
	Number	Percent	Number	Percent	Number	Percent		
C84 × C94	10	24.4	24	58.5	7	17.1	41	Not linked
C84 × C140	13	30.5	27	45.8	14	23.7	59	Not linked
C84 × C141	20	24.1	47	56.7	16	19.2	83	Not linked
C84 × T1710	6	22.2	13	48.2	8	29.6	27	Not linked
C94 × C140	104	88.1	13	11.0	1	0.9	118	Linked
C94 × C141	22	29.0	48	63.2	6	7.8	76	Not linked
C94 × T1710	113	85.6	19	14.4	0	0.0	132	Linked
C140 × C141	23	22.0	62	59.6	19	18.4	104	Not linked
C140 × T1710	135	100.0	0	0.0	0	0.0	135	Linked
C141 × T1710	24	25.5	63	67.1	7	7.4	94	Not linked

* 8H = asci having 8 histidineless spores; 6H–2+ = asci having 6 histidineless spores and 2 wild-type spores; 4H–4+ = asci having 4 histidineless spores and 4 wild-type spores.

the genetic evidence suggests that the mutant genes of C140 and T1710 are alleles. It should be noted, however, that observations of aborted spore patterns (McClintock 1945) with crosses involving T1710 indicate the existence of a chromosomal aberration in this strain. As will be described later in this report, evidence indicates that mutants T1710 and C140 are physiologically different. Thus the four mutants C84, C94, C140 and C141 are genetically different and T1710 may represent a fifth type. Mutants C84, C141 and those of the linked group (C94, C140 and T1710) have been crossed to marker mutants and the histidineless mutants placed in the linkage groups described by Houlahan *et al.* (1949). Pertinent data are included in table 2. Strain C84 can thus be placed in group *E*, C141 in group *D*, and the remaining three strains in group *A*.

In the course of the genetic experiments described above double histidineless mutants involving C84, C94, C140, C141 and T1710 were isolated. All

were verified by outcrossing them to wild type and examining asci. Any ascus from such an outcross which has a 6 histidineless–2 wild or an 8 histidineless composition confirms the fact that the histidineless parent is a double mutant.

From each outcross of the double mutants the spores of several asci having the 6 histidineless–2 wild composition were transferred to tubes (containing basal agar medium supplemented with histidine) and retained for other tests.

Accumulations

By application of the Pauly reaction for detection of histidine, as modified by JORPES (1932), it has been demonstrated that mutants C84, C85, C91, C141 and T1710 accumulate in the culture medium substances that appear to be imidazole derivatives. C94 and C140 show no significant accumulations

TABLE 2

*Linkage of histidineless mutants to other Neurospora mutants.**

Histidine mutant	Linkage group	Marker	Wild type spores	Mutant spores	Percent wild type
C84	B	51602	381	837	31.2
C84	C	10575	454	1129	28.6
C84	E	33933	304	1387	14.2
C84	E	37401	133	3991	3.2
C94	A	35203	67	1645	3.9
C94	B or D	45502	359	959	27.2
C94	C	10575	153	400	27.6
C94	E	33933	353	1024	26.7
C141	B	75001	730	2222	24.7
C141	B	51602	1013	2787	26.6
C141	C	10575	375	1190	23.9
C141	D	37301	681	4210	13.9
C141	D	38502	320	2128	13.1
C141	E	33933	438	1396	23.9

* See HOULAHAN, BEADLE and CALHOUN (1949) for identification of linkage groups and marker mutants.

of substances that give this color reaction nor do wild type strains. All of the mutants were allowed to grow for 4 days at 25°C with a quantity of histidine that will give half maximum growth. These accumulations were confirmed by chromatographing on filter paper by the method of WILLIAMS and KIRBY (1948) culture filtrates of each of the mutants. Substances were located on the chromatographs by the AMES and MITCHELL (1951) modification of the Pauly test. The solvent used for chromatographing was 3 parts propanol to 1 part 0.2 normal ammonium hydroxide.

According to the chromatographic evidence C84, C85 and C91 all accumulate the same compound, and since genetic tests have indicated that these three mutants are due to mutations at the same locus, only C84 was tested further. The C84 compound becomes purple-red on developing and has an Rf of 0.46. C141 accumulates two compounds one of which is apparently the same as that of C84. The second compound becomes a bright red on developing and

has an Rf of 0.73. The T1710 compound becomes a bright red and has an Rf of 0.65. All of the above compounds were found to be inactive biologically when tested for growth-promoting activity on the mutants. However, they proved to be extremely valuable in determining the possible sequence of biosynthetic reactions interfered with by the mutants.

None of the mutants was observed to accumulate any substance in the medium which will stimulate the growth of any other histidineless mutant. This has been tested by incorporating the filtrate of a mutant, grown on half its maximum requirement of histidine, into basal medium and inoculating with each of the other mutants. It has also been tested by chromatographing concentrated filtrates of the mutants and eluting segments of the chromatograph with basal medium. The fractions were then tested as growth supplements for all of the mutants.

Biochemical evidence for the sequence of genes in histidine biosynthesis

Previous investigations in this laboratory have made use of double mutants to provide evidence of a biochemical sequence for Neurospora mutants that have the same growth requirement (MITCHELL and HOULAHAN 1946). In the present case it has been demonstrated that mutants C84, C141 and T1710 accumulate substances that appear to be imidazole derivatives while mutants C94 and C140 do not accumulate such substances. If the five mutants correspond to five steps in a sequence of biochemical reactions that leads to the formation of histidine, and if the substances accumulated are intermediates or closely related to intermediates, then double mutants should have the properties of the single mutant that corresponds to the earlier chemical reaction in the sequence. For example the double mutant C94/C141 should not accumulate an imidazole derivative if C94 corresponds to an earlier biochemical reaction than that of C141. In order to test the above hypothesis the following series of experiments was carried out.

The double mutants and each of the single mutants were cultured for 4 days in 125-ml flasks on 20 ml minimal medium supplemented with histidine. Since the half-maximum histidine requirement of the double mutant was unknown, two cultures of each double mutant were used. These were supplemented with the half-maximum histidine requirement of each of the single mutant parents. After four days growth at 25°C a 1-ml sample was removed from each flask and transferred to a test tube. A standard set of test tubes containing minimal medium supplemented with 25γ, 50γ, and 100γ of L-histidine HCl per ml was also prepared. The intensities of the colors from all samples, after developing with the diazo reagent, were immediately read in the Beckman spectrophotometer at a wave length of 480 mμ. The compounds accumulated by the mutants give characteristic intensities of color when developed by this test.

Another series of 1-ml samples corresponding to those used in the above test was withdrawn from the growth flasks, evaporated to 0.1 ml over sulfuric acid *in vacuo* and chromatographed in the manner previously mentioned. All

samples were placed on the same sheet of filter paper to make comparison easier.

The mycelial pad from each of the growth flasks was then removed, dried at 80°C for 5 hours and weighed to determine the growth of each mutant on each histidine level in 4 days.

TABLE 3

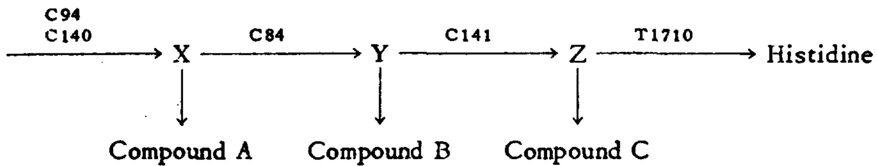
Comparison of growth and products accumulated by different histidineless Neurospora mutants.

Histidineless mutant	Amt. histidine (mg/ml) in medium before growth	Dry weight of mycelium in mg	Optical density	Compound accumulated (chromatograph)
Minimal (blank)	....	....	0.0	....
" + 25γ histidine	....	....	0.270	....
" + 50γ "	....	....	0.575	....
" + 100γ "	....	....	1.210	....
7A (wild type)	0.0045	80.9	0.000	
" " "	0.015	79.8	−0.020	None
" " "	0.030	85.5	0.000	
C84 (single)	0.0045	5.8	0.225	
" "	0.015	15.0	0.480	Type A
" "	0.030	27.5	0.480	
C94 (single)	0.0045	5.8	0.000	
" "	0.015	20.1	0.000	None
" "	0.030	35.5	0.016	
C140 (single)	0.0045	6.4	0.017	
" "	0.015	19.3	0.083	None
" "	0.030	34.1	0.090	
C141 (single)	0.0045	0.2	0.025	
" "	0.015	10.0	0.690	Types A and B
" "	0.030	18.0	1.620	
T1710 (single)	0.0045	60.5	0.250	
" "	0.015	61.8	0.182	Type C
" "	0.030	64.4	0.185	
C84/C94 (double)	0.015	18.6	0.075	None
C84/C140 "	0.015	18.0	0.072	None
C84/C141 "	0.015	0.2	0.118	Type A
" "	0.030	8.6	0.620	
C84/T1710 "	0.0045	6.6	0.160	Type A
" "	0.015	18.2	0.550	
C94/C140 "	0.015	12.9	0.071	None
C94/C141 "	0.015	18.9	0.050	None
" "	0.030	33.7	0.060	
C94/T1710 "	0.0045	5.2	0.065	None
" "	0.015	14.9	0.014	
C140/C141 "	0.015	15.6	0.034	None
" "	0.030	31.2	0.070	
C141/T1710 "	0.0045	10.4	0.275	Types A and B
" "	0.030	32.4	0.780	

The same series of experiments was repeated using a 10-day growth period. The results of the above experiments are presented in table 3. The results for the 4-day growth series are not given since they were much like those obtained at 10 days.

It will be noted in table 3 that wild-type Neurospora metabolizes all of the histidine in the medium even though this supplement is not needed for its growth. The controls also show that the histidineless mutants use all of the histidine in the medium and that their growth is dependent on the amount of histidine present.

On the basis of the results of table 3 it is postulated that the sequence of reactions interfered with by the mutants is as follows:



The results of the chromatographic and colorimetric determinations on any given double mutant are consistent in all cases with the placing of a single mutant character in the above scheme. C94 and C140 appear to correspond to biochemical reactions that come first since any double mutant made with either does not accumulate any imidazole-like substance. It is not possible to determine the order with respect to C94 and C140 since neither is an accumulator. C84 is placed before C141 and T1710 since the double mutants C84/C141 and C84/T1710 accumulate only the compound accumulated by C84. C141 is placed before T1710 since the double mutant C141/T1710 accumulates the compounds of C141 and C84. This is characteristic of the C141 single mutant. The dry weights of the mycelia show that all of the double mutants take the histidine requirement of one single, double mutant C94/T1710 that of C94, the double mutant C84/C141 that of C141, etc.

As a further check on this experiment each double mutant used previously was outcrossed to wild-type. After the spores had ripened a number of asci from each outcross were dissected on minimal agar Petri plates. The composition of each ascus as to histidineless and wild-type spores was determined as described earlier. Several asci having a 6 histidineless–2 wild spore composition were saved from every cross. One spore from each of the four spore pairs of such asci was transferred from the minimal agar plate to a test tube containing histidine-supplemented medium. Each ascus of this type provides one wild-type culture, one double mutant culture, and cultures of each of the parental single mutants. The same tests as those previously carried out with the double and single mutants were performed on each of the four spore pairs of the asci saved from the outcrosses. Several asci from each outcross except C84/C140, C84/T1710 and C94/T1710 were analyzed in this manner.

The results obtained from the above experiment demonstrate that the strains tested as double mutants (table 3) did have the expected genetic con-

stitution. The data on accumulation of imidazole-like substances by each culture from each ascus tested are completely consistent with the results shown earlier in table 3. Thus all of the data are in accord with the biochemical sequence that has been proposed. This is true even though the compounds accumulated by the single mutants do not seem to be intermediates in biosynthesis of histidine but appear to be by-products of these biosynthetic reactions.

SUMMARY

A series of 7 *Neurospora crassa* mutants which require histidine for growth have been isolated using the procedure of LEIN *et al.* (1948). These have been given isolation numbers C84, C85, C91, C94, C140, C141, and T1710.

No compound other than histidine has been found to be active in promoting growth, but different mutants require different amounts of histidine.

Growth of all of these mutants is inhibited, even when histidine is furnished, if mixtures of certain amino acids are present in the culture medium. When any one of L-leucine, DL-isoleucine, DL-valine, DL-methionine, glycine, L-tyrosine, DL-phenylalanine, L-tryptophan or histamine is present along with L-arginine, DL-ornithine, α-amino ϵ-guanidino caproic acid or L-lysine inhibition results. Tests on inhibitory concentrations of tyrosine and arginine indicate that the molar concentration of tyrosine must exceed that of histidine for complete inhibition, but if tyrosine is increased then the arginine concentrations may be lower than that of histidine.

A genetic analysis of the 7 mutants indicates that 3 different linkage groups and 4 different loci are involved. C84, C85 and C91 are in one linkage group and appear to be alleles. C94, C140 and T1710 are in another linkage group, but C94 is at a different locus from that of C140 and T1710 which act as alleles. However, the latter two mutants are biochemically different. C141 has been placed in a third linkage group.

None of the mutants has been observed to accumulate any product in the medium which is biologically active for any of the other mutants. C84, C141 and T1710 accumulate biologically inactive compounds which appear to be imidazole derivatives. These compounds are different and those mutants can be differentiated by chromatographing culture fluids on filter paper.

All double histidineless mutants were isolated from intercrosses of the single mutants. Ability of the double mutants to accumulate those compounds accumulated by the single mutants and the histidine requirements of the double mutants were used to provide evidence for a proposed biosynthetic sequence corresponding to the single mutants.

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