THE EFFECTS OF GENE CHANGE ON TRYPTOPHAN DESMOLASE FORMATION*

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Genetic and biochemical investigations have provided substantial evidence for the view that physiological reactions are under the control of nuclear genetic material. These investigations have also indicated that the inability to carry out cellular biochemical reactions is a phenotypic expression of gene change and presumably results from gene-determined variations in the formation and/or the characteristics of particular enzyme systems.

Although the existing evidence suggests a close relationship between gene, enzyme and physiological reaction, the manner in which enzymes are controlled by genes and affected by gene change is poorly understood. In an effort to define more clearly the relationship between gene and enzyme, the present investigation was initiated.

Characteristics of Mutant Strains C-83 and S-1952.—The two Neurospora mutants employed in this investigation, strains C-83[1] and S-1952,[2] are characterized by a tryptophan requirement which cannot be satisfied by indole. The biochemical reaction presumably blocked in the two mutants, the coupling of indole and serine, has been demonstrated to be the principal and probably the sole means of tryptophan synthesis in Neurospora.[3] Since the enzyme catalyzing tryptophan synthesis, tryptophan desmolase, is extractable from wild type mycelium in an active cell-free state,[4] mutants C-83 and S-1952 constitute material in which the relationship of gene to enzyme can be investigated readily.

The tryptophan requirement characteristic of mutants C-83 and S-1952 was demonstrated to be due to a single gene. From crosses of each mutant with several standard wild type strains, invariably 1:1 segregation of the tryptophan requirement was observed in all asci tested. Both the C-83 and S-1952 mutant genes are approximately the same distance from the centromere (C-83 uncorrected centromere distance = 32 based on 51 asci, S-1952 uncorrected centromere distance = 33 based on 57 asci) and are linked to the fluffy locus (fluffy locus centromere distance = 29).[5] Allelism tests were performed with the two mutants by Dr. Dorothy Newmeyer of Stanford University and of 6300 ascospores obtained by crossing the mutants to each other, no tryptophan independent ascospores were recovered. Attempts in this laboratory to form tryptophan independent heterocaryons between the two mutants were also unsuccessful. Preliminary reversion experiments employing ultraviolet light as a mutagenic

agent indicate that both mutant genes revert to tryptophan independence (T^+) at about the same frequency (C-83, 43 $T^+/2.9 \times 10^7$ viable macroconidia; S-1952, 44 $T^+/1.8 \times 10^7$ viable macroconidia). Together these observations suggest that the C-83 and S-1952 mutant genes (henceforth designated as td_1 and td_2, respectively) are either allelic or closely linked. In the absence of contradictory evidence, they will be considered alleles in this paper. However, further experiments are in progress which are designed to examine more fully the linkage relationships between the two mutant genes and the nature of the reversions to tryptophan independence.

Since both mutants are blocked in the conversion of indole to tryptophan their culture filtrates were tested for possible indole accumulation. Indole (tentatively identified on the basis of color reactions with *p*-dimethylaminobenzaldehyde and xanthydrol) and anthranilic acid as well were present in culture filtrates from both strains. Mutant S-1952 was consistently found to accumulate several times as much indole as did mutant C-83. Whether or not this can be used as a distinguishing characteristic is difficult to tell at present since tryptophan dependent cultures obtained by crossing the two mutants to wild type strains show many different levels of indole accumulation. However, strains with the td_2 allele always accumulate more indole than do strains with the td_1 allele.

The nutritional behavior of mutants C-83 and S-1952 suggests that both strains lack the enzyme present in wild type strains which catalyzes tryptophan synthesis from indole and serine. To obtain direct evidence bearing on this possibility experiments were performed to determine whether or not extracts from mutant mycelium contain tryptophan desmolase activity. These tests were carried out with extracts and preparations from mutant mycelium which was grown under various culture conditions. The methods employed in obtaining tryptophan desmolase preparations are described in detail elsewhere.[6] For greater assay sensitivity a modification of the tryptophan desmolase assay procedure[6] was employed in these experiments. The incubation mixture contained 0.2 or 0.3 μM indole, 60 μM *DL*-serine, 20 μg. barium pyridoxal phosphate,[7] 0.06 ml. of 0.5 M phosphate buffer at pH 7.8 and enzyme extract in a 1 ml. volume. Assay tubes were generally incubated for two hours at 37°. At the end of this period 0.2 ml. of 5% NaOH was added to each tube to stop the reaction. Following the addition of base, 4 ml. of toluene were added to each tube. The tubes were then shaken and the layers allowed to separate. Aliquots removed from the toluene layer were assayed for indole,[8] and when desired, tryptophan was determined[9] in aliquots of the lower layer. The assay conditions employed are capable of detecting $^1/_{400}$ of the amount of tryptophan desmolase activity present in extracts from wild type strains.

Table 1 presents a summary of the tests carried out with mutant extracts and with various fractions obtained from these extracts. In addition to these tests, mutant extracts were incubated in the presence of pyridoxamine phosphate, yeast extract, casein hydrolysate, and with various

TABLE 1

PREPARATIONS FROM MUTANTS C-83 AND S-1952 EXAMINED FOR TRYPTOPHAN DESMOLASE ACTIVITY

STRAIN	GROWTH SUPPLEMENT	GROWTH TEMP.	AGE OF MYCELIUM, DAYS	METHOD OF EXTRACTION	FRACTIONS TESTED
C-83	Optimal T	25°	2, 3, 4, 5	Homogenized in 0.1 *M* phosphate at pH 7.8	CE, DCE
		30°	3, 4, 5		
C-83	Optimal T	30°	3	Homogenized in 0.1 *M* phosphate at pH 7.8	Cell debris CE, 30%, 50%, 70% $(NH_4)_2SO_4$ ppts.
C-83	Limiting T Optimal T Limiting T + CH Optimal T + CH	30°	3	Homogenized in 0.1 *M* phosphate at pH 7.8	CE, DCE, PP, DPP
C-83	Limiting T + S	25° shaken	4	Homogenized in 0.1 *M* phosphate at pH 7.8	CE, PP
C-83	Optimal T + S	30°	3	Mycelium extracted with water and assayed at various pH's	CE
C-83	Optimal T	30°	3	Extract of lyophilized mycelium	Cell debris CE, DCE
S-1952	Optimal T Limiting T Limiting T + S	30°	2, 3	Homogenized in 0.1 *M* phosphate at pH 7.8	CE, DCE, PP
S-1952	Limiting T + S	25° shaken	4	Homogenized in 0.1 *M* phosphate at pH 7.8	CE, PP
C-83-*su*	Optimal T Optimal T + S	30°	3	Homogenized in 0.1 *M* phosphate at pH 7.8	CE, DCE, PP, DPP

T = Trypotophan. S = Serine. CE = Crude extract. CH = Casein hydrolysate. DCE = Dialyzed crude extract. PP = The partially purified preparation obtained by fractionating a crude preparation according to the procedure used with wild type extracts. DPP = Dialyzed purified preparation.

concentrations of indole, serine and pyridoxal phosphate. Assays were also performed at several temperatures. Tryptophan desmolase activity could not be detected in mutant extracts under any conditions nor could activity be detected in any fraction obtained from these extracts. Mutant extracts were also mixed with wild type tryptophan desmolase prepara-

tions to test for the presence of inhibitors and no appreciable stimulation or inhibition was observed.

These results are in agreement with those obtained previously by Mitchell and Lein[10] in a study of one of these mutants, C-83. They too were unable to detect tryptophan desmolase activity in extracts from this mutant. The only report of the presence of tryptophan desmolase activity in mutant C-83[11] has been retracted[12] as a result of further experimentation. At the present time, therefore, it appears that neither extracts from strain C-83 nor from strain S-1952 contain tryptophan desmolase activity which can be detected by the methods employed.

Although tryptophan desmolase activity could not be demonstrated in mutant extracts, it is conceivable that there is active desmolase in the mutants and that the enzyme is destroyed during extraction or is inactive under the assay conditions employed. To obtain some data concerning

TABLE 2

CROSSES INVOLVING THE SUPPRESSOR GENE

	CROSS	ASCUS TYPE 8T+	6T+:2T−	4T+:4T−
1.	Chilton wild type × S-1952 "reverted"	1	6	2
2.	Lein wild type × S-1952 "reverted"	1	8	3
3.	Chilton wild type carrying the suppressor × S-1952	1	8	2
4.	Lein wild type carrying the suppressor × S-1952	3	11	3
	Total	6	33	10
5.	Chilton wild type carrying the suppressor × C-83	0	0	5
6.	Lein wild type carrying the suppressor × C-83	0	0	5
	Total	0	0	10

T^+ = Tryptophan independent. T^- = Tryptophan dependent.

these possibilities an isotope experiment was performed to determine whether mutant C-83, when growing, is capable of tryptophan synthesis. The details of this experiment have been published previously.[13] In brief, mutant C-83 was grown on N^{15} ring labeled tryptophan and after maximum growth was attained tryptophan was isolated from the mycelium formed. The isolated tryptophan had the same N^{15} content as the labeled tryptophan fed, showing that mutant C-83 did not synthesize a significant amount of tryptophan under the conditions of this experiment.

These data, then, all support the view that mutants C-83 and S-1952, biochemically similar in their behavior, are both unable to synthesize tryptophan because of the absence of a normally essential enzyme, tryptophan desmolase. Thus these two mutants would appear to represent strains in which the result of gene mutation is the loss of a particular enzymatic activity.

Suppressor Studies.—During the course of this work a microconidial

stock of strain S-1952 reverted to tryptophan independence. Microconidia from this reverted culture were plated out and a homocaryotic tryptophan independent culture was recovered. This strain was then crossed with two standard wild type strains and as can be seen in table 2 (crosses 1 and 2), three types of asci were obtained. The most frequent ascus class had 6 tryptophan independent and 2 tryptophan dependent ascospores. This finding suggests that the reversion to tryptophan independence was due to a genetic change at a separate locus (*su*). This change appears to result in the suppression of the tryptophan requirement associated with the td_2 allele. As a further check of this conclusion tryptophan independents recovered from 4:4 asci ($4T^+:4T^-$) were crossed to mutant S-1952 and again three types of asci were obtained (table 2, crosses 3 and 4).

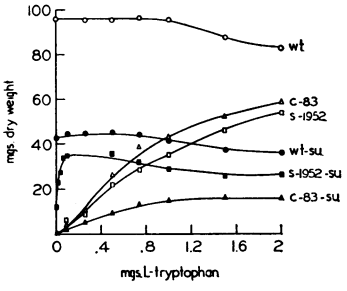

FIGURE 1

Growth of various strains of Neurospora on different levels of tryptophan. Incubated for 72 hours at 30°.

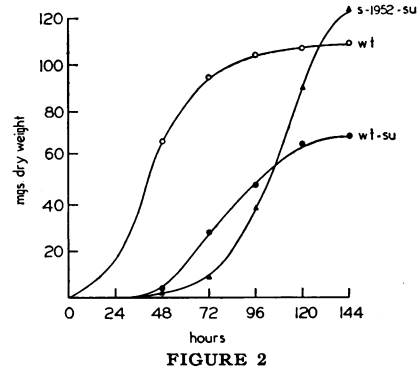

FIGURE 2

Growth curves of various strains on minimal medium at 30°.

The cultures obtained from these crosses were scored by inoculating two flasks with conidia, one flask containing minimal medium and the other flask containing minimal medium plus 1 mg. *L*-tryptophan. These flasks were incubated for 72 hours at 30° after which the mycelial pads were removed, dried and weighed. By employing this procedure it is possible to distinguish the four possible types of cultures: mutant, suppressed mutant, wild type and wild type carrying the suppressor gene. The mutant and wild type cultures can easily be identified since the former cultures do not grow on minimal medium while the latter cultures grow well on both types of media. The other two types of tryptophan independent cultures can be identified also, since the suppressed mutant grows poorly on minimal medium (in a 72-hour period) and better in the presence of tryptophan, while the growth of wild type strains carrying the suppressor gene is poor on the same two media and is not stimulated by

tryptophan. Comparisons of the growth of the various types of strains are shown in figures 1 and 2. In figure 2 it is seen that there is a lag in the growth of strains carrying the suppressor gene. Attempts to overcome this lag or inhibition associated with the suppressor gene by adding various supplements have been unsuccessful.

To obtain some information concerning the specificity of the suppressor gene, wild type strains carrying this gene were crossed to mutant C-83. As may be seen in table 2 (crosses 5 and 6) only 4:4 asci ($4T^+:4T^-$) were obtained. Thus the effect of the suppressor gene appears to be specific for the td_2 allele. That some of the tryptophan dependent cultures derived from these crosses actually carry the suppressor gene was demonstrated by performing crosses with strain S-1952. Additional crosses are being performed to determine if a particular genetic background is necessary for the suppressor gene to show its effect on the td_1 allele. Green[14] has also reported a case in which a suppressor gene does not affect every member of an allelic series

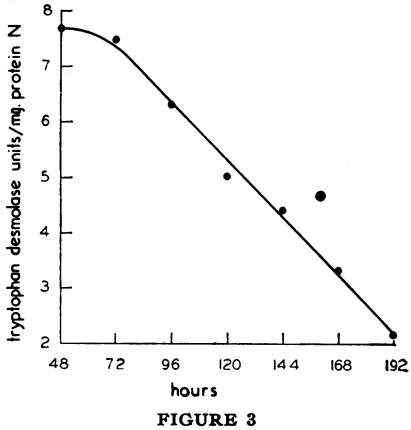

FIGURE 3

Variation in the tryptophan desmolase content of wild type mycelium with time. For the unit of enzyme activity see (6).

C-83 strains carrying the suppressor gene can be distinguished from C-83 strains without this gene since the presence of the suppressor gene results in a marked inhibition of growth (see Fig. 1). It can also be seen in figure 1 that C-83 strains require less tryptophan to reach maximum growth when they carry the suppressor gene. However, since the growth of strains with the td_1-*su* genotype is poor under all conditions, it is difficult to evaluate this observation.

Examination of Strains Carrying the Suppressor Gene for Tryptophan Desmolase Activity.—Since S-1952-*su* strains grow in the absence of exogenous tryptophan, enzyme studies were performed to see if this ability could be correlated with the presence of tryptophan desmolase. Also examined for tryptophan desmolase activity were two wild type strains carrying the suppressor gene and a C-83 strain carrying the suppressor gene (tryptophan dependent). Tryptophan desmolase activity was present in extracts from strain S-1952-*su* as well as in extracts from wild type strains carrying the suppressor gene. No activity could be detected in extracts from strain C-83-*su*. Attempts to activate extracts from this latter strain were also unsuccessful (see table 1). Thus the suppressor gene when present with

the td_2 allele restores both the ability to synthesize tryptophan desmolase and to grow in the absence of exogenous tryptophan; neither of these effects is observed in strains with the td_1-*su* genotype.

An effort was made to compare the amount of tryptophan desmolase in strain S-1952-*su* with the amount in wild type strains with and without the suppressor gene. The tryptophan desmolase content of wild type mycelium varies considerably with time as can be seen in figure 3 and, in view of the growth behavior of strains S-1952-*su* and wild type carrying the suppressor gene (see Fig. 2), such a comparison is difficult. Accepting these uncertainties, the maximum amount of tryptophan desmolase found in a suppressed S-1952 strain was approximately $1/_{20}$ of the maximum amount of tryptophan desmolase that is formed by wild type strains.

TABLE 3

COMPARISON OF THE EFFECT OF VARIOUS INHIBITORS ON TRYPTOPHAN DESMOLASE FROM STRAIN S-1952-*su* AND FROM A WILD TYPE STRAIN

ADDITION	FINAL CONCENTRATION, MOLAR	% INHIBITION	
		SUPPRESSED MUTANT DESMOLASE	WILD TYPE DESMOLASE
Hydroxylamine	1×10^{-3}	100	92
F^-	1×10^{-3}	2	3
Co^{++}	1×10^{-3}	73	76
CN^-	1×10^{-3}	40	54
Zn^{++}	1×10^{-3}	78	81
Mg^{++}	1×10^{-3}	0	0
NH_4Cl	0.19	29	23
NH_4Cl	0.37	44	46
L-tryptophan	1×10^{-3}	22	19
L-tryptophan	2×10^{-3}	36	29

Thus the presence of the suppressor gene restores to strain S-1952 the ability to form effective concentrations of tryptophan desmolase but does not permit tryptophan desmolase synthesis to proceed to the same extent as it does in wild type strains. The stimulation of growth of strain S-1952-*su* by low concentrations of tryptophan (see Fig. 1) is in agreement with this finding. Wild type strains carrying the suppressor gene have a maximum tryptophan desmolase content which is about one-third that of wild type strains without the suppressor gene.

Comparison of Wild Type Tryptophan Desmolase and Tryptophan Desmolase from Strain S-1952-su.—Several characteristic properties of the tryptophan desmolase from a wild type strain were next compared with the properties of tryptophan desmolase from strain S-1952-*su*. On a molar basis indole uptake and tryptophan formation are equivalent in the presence of preparations from either strain. The stability of both preparations is roughly the same, both losing approximately 50% of their trypto-

phan desmolase activity when stored at 2° for 24 hours. Both preparations are inhibited by the same substances, and to practically the same extent (see table 3). As was the case with wild type preparations,[6] indole was not taken up by suppressed-mutant tryptophan desmolase preparations in the presence of the following substitutes for serine: alanine, glycine, cysteine, cystine, methionine, threonine, aspartate, glutamate, acetate, pyruvate, succinate or malate.

Before other comparisons were performed an attempt was made at fractionation and partial purification of suppressed-mutant tryptophan desmolase preparations according to the procedure developed for wild type preparations.[6] This procedure involves the use of various concentrations of ammonium sulfate at two pH values. Suppressed-mutant preparations and wild type preparations behaved identically throughout the steps in this procedure. The partially purified (about 20-fold) suppressed-mutant tryptophan desmolase preparations which were obtained were then employed in further comparisons. The pH-activity curves for such a suppressed-mutant preparation and for a wild type preparation are shown in figure 4. Both preparations exhibit maximum activity at pH 7.8.

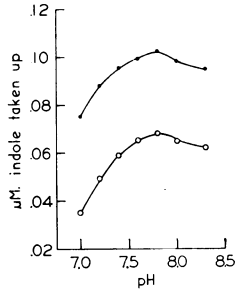

FIGURE 4

pH-activity curves for tryptophan desmolase preparations from a wild type strain (●—●) and from strain S-1952-*su* (○—○). 1 hour incubation at 37°. 2×10^{-4} *M* indole, 6×10^{-2} *M* DL-serine, 20 μg. barium pyridoxal phosphate per ml.

Comparisons of the affinity of the two partially purified enzyme preparations for their substrates and for their coenzyme were also performed. In the determinations of the Michaelis constant for serine, excesses of indole and pyridoxal phosphate were employed and indole uptake was measured. A Lineweaver-Burk plot[15] of kinetic data for serine is shown in figure 5. The approximate K_m value for serine for both preparations can be estimated from figure 5 to be 3.4×10^{-3} *M*. This value agrees fairly well with the value of 6×10^{-3} *M* found for wild type tryptophan desmolase preparations under different conditions.[6] Pyridoxal phosphate saturation curves are shown in figure 6. The pyridoxal phosphate concentration required to restore half-maximum activity is approximately the same for the two enzyme preparations, 3×10^{-6} *M*. It can also be seen from figure 6 that neither enzyme preparation (both were partially purified) was active in the absence of pyridoxal phosphate. The velocity of indole uptake by both preparations is essentially zero order at indole concentrations higher than 2×10^{-4} *M*.

The energy of activation was determined for partially purified wild type and suppressed-mutant preparations. Excesses of indole and

pyridoxal phosphate and a limiting concentration of serine were employed. Indole uptake was measured and the corresponding decrease in serine concentration was calculated and is plotted in figure 7 (*a*). The velocity constants obtained from these curves were then used to determine the energy of activation. The log k against $1/T$ plot is shown in figure 7 (*b*). In this figure it can be seen that the points obtained with the two enzyme preparations fall on the same line. The energy of activation, therefore, is approximately 12,000 cal./mole. in each case.

Thus the tryptophan desmolase preparations obtained from both strains appear to be similar by the criteria applied.

Discussion.—The data presented in this paper permit certain conclusions concerning the relationship between gene and enzyme. The studies with

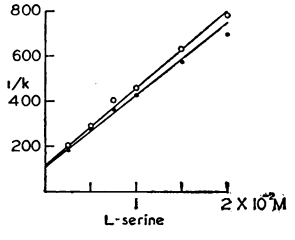

FIGURE 5

Lineweaver-Burk plot of kinetic data for serine. 1 hour incubation at 37°. Wild type tryptophan desmolase (●—●) S-1952-*su* tryptophan desmolase (O—O). 3×10^{-4} *M* indole, 20 μg barium pyridoxal phosphate per ml.

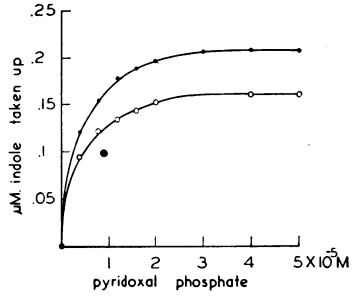

FIGURE 6

Pyridoxal phosphate saturation curves for tryptophan desmolase preparations from a wild type strain (●—●) and from strain S-1952-*su* (O—O). 1 hour incubation at 37°. 3×10^{-4} *M* indole, 6×10^{-2} *M* DL-serine.

mutants C-83 and S-1952 have shown that genetic alteration has affected the ability of each strain to produce active tryptophan desmolase. The nature of the effect on tryptophan desmolase or tryptophan desmolase formation which is responsible for the absence of detectable activity cannot be stated. It is clear, however, that if tryptophan desmolase is present in the mutants it does not provide sufficient tryptophan to support growth.

Although strains with the td_2 allele (S-1952) lack tryptophan desmolase activity, they are capable of forming active tryptophan desmolase when they are carrying a specific form of a different gene. This second gene, however, although it enables strains with the td_2 allele to form active enzyme, does not appear to be capable of mediating tryptophan desmolase

synthesis by itself. This conclusion is drawn from the observation that strains with the td_1-*su* genotype do not have tryptophan desmolase activity. In strain S-1952-*su* then, two genes, both in specific forms, appear to be required for the synthesis of tryptophan desmolase.

In addition to this case in which two specific genes appear to be required for the formation of a single enzyme, there are other cases in which mutation of any one of a number of genes affects the formation of single enzymes.[16–18] Together, these findings might be interpreted as indicating that the relationship between genes and enzymes is exceedingly complex. However, since so many cellular reactions are interrelated and interdependent it is difficult to tell at which level the complexity exists.

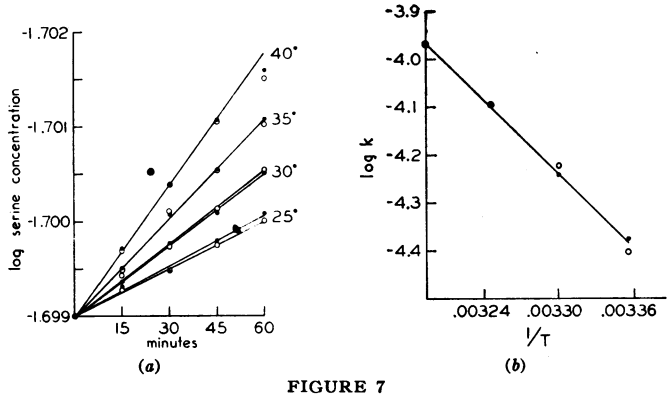

FIGURE 7

The effect of temperature on the rate of serine uptake (indole uptake was measured) by tryptophan desmolase preparations from a wild type strain (O—O) and from strain S-1952-su (●—●). 2×10^{-4} *M* indole, 4×10^{-2} *M* DL-serine, 30 μg. barium pyridoxal phosphate per ml.

In view of the fact that two genes, each differing from its wild type allele, are required for tryptophan desmolase synthesis in strain S-1952-*su*, one might expect the tryptophan desmolase formed by this strain to have altered properties. In a preliminary comparison in which a number of enzyme characteristics were examined, no major difference was found between the tryptophan desmolase extracted from strain S-1952-*su* and the same enzyme extracted from a wild type strain. To be sure, the methods employed could not reveal all types of changes that could occur in the suppressed mutant desmolase, nor are they sensitive enough to detect very slight changes. However, the catalytic portion of S-1952-*su* tryptophan desmolase, at least, does not appear to be altered appreciably.

The question of the effect of gene change on enzyme specificity has been

considered in other investigations[16-19] and, as yet, there is no clear demonstration of a change in enzyme specificity as a result of gene mutation. The recent findings of Pauling, *et al.*,[20] however, do suggest that such changes may occur. In any case, since relatively few studies of the effects of gene change on enzyme specificity have been performed, we cannot exclude alteration of enzyme specificity as a possible result of gene mutation.

It is difficult to account for the fact that the suppressor gene permits mutant S-1952 to grow in the absence of an exogenous supply of tryptophan and to form apparently normal tryptophan desmolase. The suppressor gene does not appear to be a duplication at another locus of the normal allele of the S-1952 locus since strains with the td_1-*su* genotype lack tryptophan desmolase activity. Nor does it seem likely that the suppressor gene acts by creating an alternate biosynthetic pathway of tryptophan formation or by relieving strain S-1952 from a tryptophan desmolase inhibitor. Although it is relatively easy to rule out most suggested explanations at the substrate level, at levels closer to the gene many hypotheses are consistent with the results obtained. Thus, the suppressor gene could act by creating an alternate biosynthetic pathway of tryptophan desmolase formation, by removing or inhibiting a reaction competing for a common enzyme precursor, by restoring the specificity of tryptophan desmolase, tryptophan desmolase precursors or the tryptophan desmolase forming system, by increasing the concentration of tryptophan desmolase precursors, tryptophan desmolase forming system or both or by affecting in any of a number of ways the rate of formation or time of appearance of tryptophan desmolase. It thus becomes apparent that we must have a better understanding of the processes involved in enzyme formation if we are to explain the mechanism of gene action.

The results obtained in this study also demonstrate that the td_1 and td_2 alleles behave differently. This can be seen from the fact that the suppressor gene, in contrast to its effect on strains with the td_2 allele, does not enable strains with the td_1 allele to grow in the absence of an exogenous supply of tryptophan, nor does it permit these strains to form detectable tryptophan desmolase. Thus the behavior of the td_1 and td_2 alleles in the presence of the suppressor gene suggests that these alleles may be qualitatively different forms of the same gene. In view of this and since there are several cases in which the complexity of single genetic loci has been demonstrated,[21-27] it is tempting to speculate that each gene may be composed of one or more units each of which is concerned with different steps in the synthesis of a single enzyme.

Summary.—Two allelic tryptophanless Neurospora mutants, both unable to use indole for growth, were examined for tryptophan desmolase, the enzyme which catalyzes tryptophan synthesis from indole in wild type strains. Tryptophan desmolase activity could not be detected in

extracts or in various preparations from either strain. One of these mutants, S-1952, does form tryptophan desmolase and grow in the absence of an exogenous supply of tryptophan when it is carrying a specific suppressor gene. This suppressor gene does not similarly affect the tryptophanless strain (C-83) with the allelic mutant gene. Since C-83-*su* strains do not form tryptophan desmolase, it appears that both the S-1952 mutant gene and the suppressor gene are required for tryptophan desmolase synthesis in strain S-1952-*su*. The tryptophan desmolase formed by a S-1952-*su* strain was compared with the tryptophan desmolase of a wild type strain and no major differences were detected.

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