GENETIC CONTROL OF A MULTIENZYME COMPLEX: SUBUNIT STRUCTURES OF MUTATIONALLY ALTERED FORMS

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IN *Neurospora crassa*, anthranilate synthetase, N-(5′-phosphoribosyl) anthranilate (PRA) isomerase, and indole-3-glycerol phosphate (InGP) synthetase are organized in a multienzyme complex whose structure and function are jointly controlled by the *tryp-1* and *tryp-2* loci (DEMoss and WEGMAN 1965; DEMoss, JACKSON and CHALMERS 1967). The wild-type complex has a molecular weight of 240,000 and is composed of six subunits of two distinct types (GAERTNER and DEMoss 1969). It was proposed that these subunits are the gene products of the *tryp-1* and *tryp-2* loci and that complex formation is an obligate step in the expression of anthranilate synthetase. This suggestion is supported by the finding that an active complex with a sedimentation constant of 10S can be produced *in vitro* by mixing extracts of certain *tryp-1* and *tryp-2* mutants (ARROYO-BEGOVICH and DEMoss 1969).

Mutations at both loci independently affect the three catalytic activities and the overall integrity of the complex (DEMoss, JACKSON and CHALMERS 1967). To understand the relationship between structure and activity we have isolated, by genetic methods, various mutationally altered gene products and have studied their interaction with wild-type subunits *in vitro*. The results demonstrate that the expression of anthranilate synthetase is dependent upon the specific association of the products of the *tryp-1* and *tryp-2* genes and have allowed us to infer the subunit structures of the altered complexes in *tryp-1* and *tryp-2* mutants.

MATERIALS AND METHODS

The strains used in this investigation are from the culture collection of this laboratory. With the exception of 75001 (*tryp-2*, FGSC #511), all mutants were induced in wild types 74A, 74-OR23-1A, or 74AF, either in this laboratory or in that of DR. ANN LACY and crossed to 74-OR8-1a. The *tryp-1* and *tryp-2* single mutants were identified and classified according to procedures described elsewhere (DEMoss and WEGMAN 1965; CHALMERS 1968).

tryp-1; *tryp-2* double mutants were constructed by crossing alleles and analyzing random spore isolates. Double mutants were identified by their inability to complement in heterocaryons with either parent but with an isogenic *tryp-3* tester strain. Confirmation was achieved by backcrossing the putative double mutants to wild type and analyzing the progeny by nutritional and complementation tests (CHALMERS 1968).

Stock cultures were maintained on minimal medium (VOGEL 1964), supplemented with 150 μg L-tryptophan per ml and 1.5% agar. Bulk mycelium for enzyme studies was grown in liquid medium plus 150 μg L-tryptophan or 20 μg indole per ml. Extracts were prepared according to

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DEMOSS (1965) and CHALMERS (1968). All extracts and sucrose gradients were made in 0.05M potassium phosphate buffer at pH 7.0, containing 10^{-4}M EDTA and 2×10^{-4}M dithiothreitol. Concentrated ammonium sulfate fractions were made by adding solid ammonium sulphate to 50% of saturation and suspending the precipitate in 0.1 of the original volume. For assays, this extract was either passed over G-25 Sephadex or diluted at least ten-fold with buffer to reduce the salt concentration.

Anthranilate synthetase was assayed fluorometrically by the method of DEMOSS (1965). PRA isomerase was determined by the technique described in DEMOSS, JACKSON and CHALMERS, (1967). InGP synthetase was measured according to DEMOSS and WEGMAN (1965). Activity is expressed in units defined as that amount of enzyme which converts or produces 1 μmole of substrate or product in one hour at 37°C. Protein was estimated by the assay of LOWRY *et al.* (1951).

Complex formation *in vitro* was performed according to the methods of ARROYO-BEGOVICH and DEMOSS (1969). *tryp-2*(6)A was used as a source of the *tryp-1* gene product, while *tryp-1*(10)A served for the *tryp-2* product. *In vitro* complementation activity is expressed as units of anthranilate synthetase activity formed in 20 min at 20°C. Crude extracts, sucrose gradient fractions, and diluted ammonium sulfate fractions could be assayed quantitatively by this method.

Zone centrifugation in sucrose density gradients was done as described in DEMOSS, JACKSON and CHALMERS (1967), using crystalline catalase (Worthington) as an internal marker at 11S. Ten-drop fractions were collected and diluted with buffer to 2 ml. Gradient fractions were routinely assayed only for InGP synthetase and anthranilate synthetase. In several cases, PRA isomerase was determined and found to be coincident with the InGP synthetase activity. In cases of very low activity, undiluted fractions were assayed directly for anthranilate synthetase and *in vitro* complex formation.

Crosses were carried out according to the technique of KAPLAN, SUYAMA and BONNER (1964) on cornmeal agar supplemented with 500 μg of L-tryptophan per ml.

RESULTS

In our earlier studies (GAERTNER and DEMOSS 1969; ARROYO-BEGOVICH and DEMOSS 1969), several lines of evidence suggested that the wild-type complex is formed *in vitro* from the unassociated gene products of the *tryp-1* and *tryp-2* loci. The two interacting components in this reaction were an enzymatically inactive 4.5S component, present in a *tryp-1* mutant, and a 7.5S component possessing PRA isomerase and InGP synthetase activity accumulated by a *tryp-2* mutant. These two components were essentially identical in their respective properties to two subunits generated from the isolated wild-type complex by a pCMB treatment. The two derived subunits were a dimer and a tetramer composed of distinct polypeptide chains and were assumed to be the products of the *tryp-2* and *tryp-1* genes, respectively. The assembly of the complex from its subunits may be followed by anthranilate synthetase formation and since the active complex does not readily dissociate, this reaction provides a sensitive assay for the presence of either unassociated subunit when the other subunit is supplied in excess. As a first step in determining the structure of the altered mutant complexes we examined various *tryp-1* and *tryp-2* mutants for the presence of unassociated subunit by this assay.

The *tryp-2* mutants studied were selected as representative of the mutant classes previously described at this locus (DEMOSS, JACKSON and CHALMERS 1967). Although all *tryp-2* mutants lack anthranilate synthetase, they may be assigned to two classes (7S tryp-2, 10S tryp-2) according to the sedimentation

TABLE 1

Classes of tryp-2 *mutants*

Strains	Estimated sedimentation constant of PRA isomerase and InGP synthetase*
tryp-2 (2)A, (3)A, (6)A, (10)A, (11)A, (13)A, (14)A, (15)a, (17)a, (19)a, (20)a, (21)a, (22)a, (26)A, (29)A, (30)A, (33)A, (36)A, (37)A, (38)A, (39)A, (40)A, (41)A, (43)A, (44)A, (46)A, (47)A, (48)a, (50)a, (51)a, (55)A, (75001)A	7S
tryp-2 (8)A, (28)A, (31)A, (42)A, (56)A	10S

* Estimated by comparing peak of activity to position of catalase markers as described in METHODS

constant of the PRA isomerase and InGP synthetase remaining. Of 37 independently isolated *tryp-2* mutants, 32 had the 7S form of PRA isomerase and InGP synthetase, while the remaining 5 had only the 10S enzyme (Table 1). Complementation tests were carried out in heterocaryons with many of the *tryp-2* mutants including both 10S and 7S strains. In agreement with CATCHESIDE (1964), no complementation was observed. These strains have been mapped by two-point crosses to a set of standard mutants (CHALMERS 1968). The maximum prototroph frequency measured was 0.19%. These data indicate that the *tryp-2* locus is a single cistron controlling both anthranilate synthetase activity and the state of association of the complex.

Mutants of the 7S class accumulated high levels of the normal *tryp-1* gene product measured by the *in vitro* formation of anthranilate synthetase (Table 2), showing that in these strains the mutant product of the *tryp-2* locus does not associate with or otherwise mask the wild-type tryp-1 subunit. On the other hand, the 10S mutants had essentially no pools of free *tryp-1* gene product by this assay. Apparently the products of both loci are associated in a stable complex and the normal tryp-1 subunit present in these *tryp-2* mutants is unavailable for interaction with the added wild-type tryp-2 subunit.

From the *tryp-1* mutants previously reported (DEMOSS, JACKSON and CHALMERS 1967), we selected a number from each of the major classes for analysis. Five of those mutants lacking all three activities of the complex (*tryp-1Z*) were examined by the *in vitro* association assay for the presence of unassociated *tryp-2* gene product (Table 2). These strains all had large amounts of the normal tryp-2 subunit, indicating that *tryp-1Z* mutants fail to make a *tryp-1* product which can effectively bind the tryp-2 subunit. The *tryp-1* strains retaining only anthranilate synthetase (*tryp-1A*) have an abnormally labile enzyme with a sedimentation constant of 7S in sucrose gradient (CHALMERS 1968). Mutants of this class possess lower levels of the *tryp-2* gene product when measured by the increase in anthranilate synthetase activity in the *in vitro* assay (Table 2). Either these mutants produce reduced amounts of normal *tryp-2* product or most of the

TABLE 2

Biochemical properties of tryp-2 *and* tryp-1 *mutants*

Class	Strain	Specific activity		
		Anthranilate synthetase*	Unassociated tryp-2 Subunit†	Unassociated tryp-1 Subunit†
tryp-2 7S	(6)A	0	....	0.30‡
	(10)A	0	....	0.14
	(75001)A	0	....	0.13
tryp-2 10S	(8)A	0	....	0.006
	(28)A	0	....	0.006
tryp-1A	(4)A	0.007	0.06	....
	(16)A	0.029	0.10	....
	(17)A	0.010	0.075	....
	(19)A	0.018	0.09	....
	(24)A	0.018	0.09	....
tryp-1Z	(8)A	0	0.17	....
	(9)A	0	0.17	....
	(10)A	0	0.19	....
	(14)A	0	0.11	....
tryp-1C	(C6)A	0	0.005	....

* Units are μmoles anthranilate produced/mg protein/hour in crude extracts.
† Assayed as anthranilate synthetase produced in the *in vitro* association assay. Activity is expressed as units of anthranilate synthetase formed per mg protein.
‡ Measured on a fraction concentrated 3-fold by ammonium sulfate precipitation.

tryp-2 product is present in the 7S anthranilate synthetase complex and unavailable for *in vitro* complex formation. The PRA isomerase and InGP synthetase activity in a *tryp-1* mutant lacking only anthranilate synthetase (*tryp-1C*) was in a 10S form (Figure 1); this mutant exhibited virtually no pool of free tryp-2 subunit (Table 2). The situation in this mutant is analogous to the 10S *tryp-2* strains in that both classes have stable 10S complexes apparently defective only in the anthranilate synthetase reaction.

From the above results we conclude that the *tryp-1Z* mutants either form no tryp-1 subunits or form products incapable of interacting with tryp-2 subunits. Therefore a *tryp-1; tryp-2* double mutant containing a *tryp-1Z* allele should accumulate the altered tryp-2 subunit in an unassociated form. In a similar way, *tryp-1; tryp-2* double mutants containing a 7S *tryp-2* allele should accumulate the altered tryp-1 subunit in an unassociated form. We have, therefore, constructed such double mutants in order to study the properties of unassociated altered subunits (Table 3). The double mutant derived from *tryp-2*(6), which makes no discernible *tryp-2* gene product, and *tryp-1*(10), which makes no discernible *tryp-1* gene product, had none of the enzyme activities of the complex, was inactive in the *in vitro* association assay with either wild-type subunit and did not interfere with the *in vitro* interaction between wild-type subunits. This result demonstrates directly that the presence of either of these alleles in a double mutant will allow a mutant allele of the opposite locus to form its product in an unassociated form.

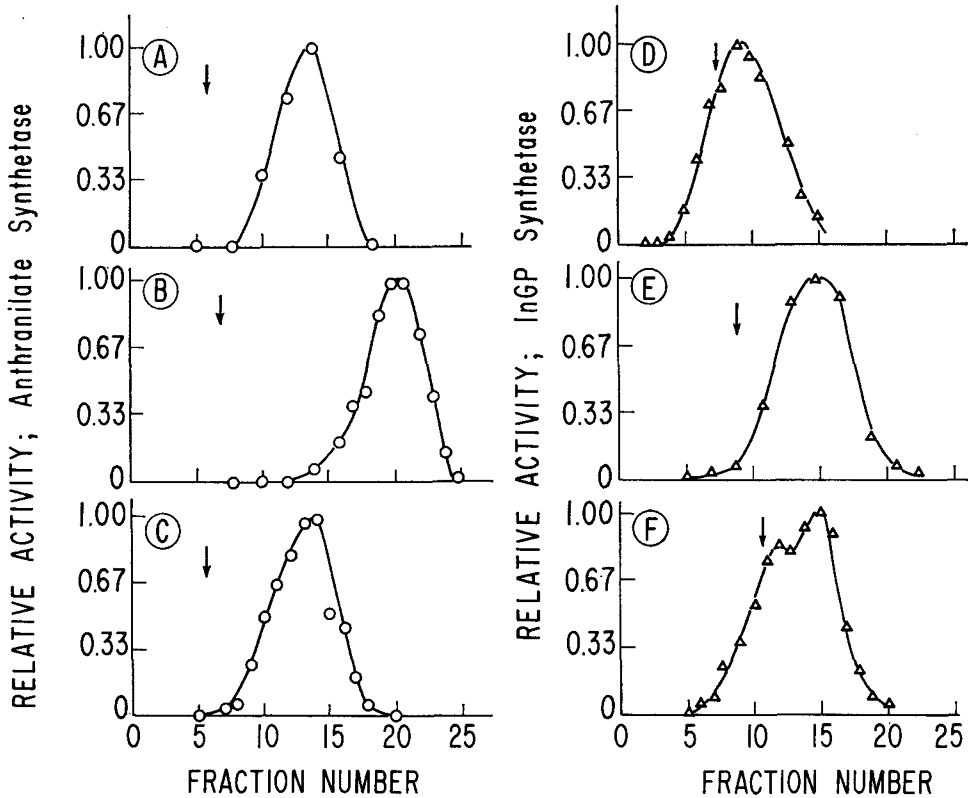

FIGURE 1.—Zone centrifugation profiles of anthranilate synthetase (O–O) and InGP synthetase (△–△). Gradients A, B, and C are analyses of *tryp-1*(17), of the *tryp-1*(17); *tryp-2*(6) double mutant, and of the *in vitro* complex formed by mixing extracts of the double mutant with wild-type tryp-2 subunit. Enzyme activities in units/ml are: A(OOO) 0.010, B(OOO) 0.009, C(OOO) 0.008. Gradients D, E, and F are, respectively, of *tryp-1*(C6), of the *tryp-1*(C6); *tryp-2*(6) double mutant, and of the *in vitro* complex formed by mixing extracts of the double mutant with limiting amounts of tryp-2 subunit. Enzyme activities in units/ml are: D(△–△) 3.3, E(△–△) 3.4, F(△–△) 2.0. The arrow indicates the position of the 11S catalase marker. The apparent sedimentation constants of the peak tubes are: A, 7S; B, 4S; C, 7S; D, 10S; E, 7S; F, 10S and 7S.

The two double mutants derived from *tryp-2*(6) and the *tryp-1A* alleles 16 and 17, had no anthranilate synthetase activity but had detectable levels of material which could interact with the normal *tryp-2* gene product to form anthranilate synthetase activity (Table 3). Figure 1 compares the zone centrifugation profiles of the anthranilate synthetase present in the parent mutant (Figure 1A), the material in the double mutant which is active in the *in vitro* association reaction (Figure 1B) and the product of the *in vitro* association reaction (Figure 1C). Because of the low activity of the double mutant extract, the extract containing the product of the *in vitro* association reaction was prepared by mixing equal parts of mycelial powders of the double mutant and of *tryp-1*(10), which contains unassociated wild-type *tryp-2* gene product, and extracting the

TABLE 3

Biochemical properties of tryp-1; tryp-2 *double mutants*

Strains	Enzyme specific activity* Anthranilate synthetase	InGP synthetase	*tryp-1*+ Gene product	Parental classes
tryp-1 (10); *tryp-2* (6)	0	0	0.000	tryp-1Z, 7S tryp-2
tryp-1 (16); *tryp-2* (6)	0	0	0.002	tryp-1A, 7S tryp-2
tryp-1 (17); *tryp-2* (6)	0	0	0.002	tryp-1A, 7S tryp-2
tryp-1 (C6); *tryp 2* (6)	0	0.3‡	0.000	tryp-1C, 7S tryp-2
tryp-1 (10); *tryp-2* (8)	0	0	—	tryp-1Z, 10S tryp-2
tryp-1 (10); *tryp-2* (28)	0	0	—	tryp-1Z, 10S tryp-2

* μmoles product/mg protein/hour
† Assayed as anthranilate synthetase formed *in vitro* in the presence of added *tryp-2* gene product. Expressed as units of anthranilate synthetase formed per mg protein.
‡ Assayed in concentrated ammonium sulphate fraction
0 indicates no measurable activity
— indicates no measurement made

mixture with buffer. The material in the double mutant which was active in forming anthranilate synthetase had a sedimentation constant of 3–4S. The anthranilate synthetase formed in this reaction was approximately 7S, similar to the anthranilate synthetase present in the parental strain.

The double mutant of *tryp-2* (6) and *tryp-1* (C6) had virtually parental levels of InGP synthetase (Table 3) and this activity banded in a sucrose gradient at approximately 7S (Figure 1E) compared to the 10S activity in the parental strain (Figure 1D). A calculated excess of the extract from the double mutant was mixed with an extract of *tryp-1* (10) (containing unassociated wild-type *tryp-2* gene product). The mixture was concentrated by ammonium sulfate precipitation and subjected to zone centrifugation. In this case no anthranilate snythetase activity was formed, but the interaction produced a 10S component with InGP synthetase activity (Figure 1F).

A similar study was carried out with two 10S *tryp-2* mutants. The double mutants produced from either *tryp-2* (8) or *tryp-2* (28) contained no InGP synthetase or anthranilate synthetase (Table 3). When extracts from these double mutants were mixed with a source of *tryp-1* gene product (*tryp-2* (6) extract), no anthranilate synthetase was produced, but the InGP synthetase originally present as the unassociated *tryp-1* gene product was shifted to the 10S region of the sucrose gradient. The altered *tryp-2* gene product present in the double mutants aggregated with the *tryp-1* gene product to form a defective 10S complex similar to the aggregate in the parental strain.

DISCUSSION

We have previously demonstrated that the wild-type complex is a hexamer of two distinct subunits and suggested that the primary structures of these subunits are controlled by the *tryp-1* and *tryp-2* loci (GAERTNER and DEMOSS 1969:

DEMOSS, JACKSON and CHALMERS 1967). From the results presented here we have deduced the subunit structures of the various altered complexes formed by *tryp-1* and *tryp-2* mutants. Our inferences as to the structure of each type of complex, based on the *in vitro* association of subunits derived from double mutants, are summarized in Table 4. In this table, we have called the products of the *tryp-1* and *tryp-2* genes the I and A components, respectively, and have represented the various complexes as combinations of these monomers or their altered forms. In this terminology the wild-type complex is A_2I_4 (GAERTNER and DEMOSS 1969).

These results directly demonstrate that the two subunits of the complex are the products of the *tryp-1* and *tryp-2* genes. In the case of the *tryp-2* 10S mutant, a mutation at the *tryp-2* locus results in the formation of an altered A subunit which still participates in complex formation but is enzymatically inactive. Likewise, specific mutations in the *tryp-1* gene (*tryp-1C* and *tryp-1A*) alter the structure of the I subunit so that an aberrant complex is formed which has only anthranilate synthetase activity (*tryp-1A*) or a 10S complex is formed which has only PRA isomerase and InGP synthetase activities (*tryp-1C*).

Furthermore, the participation of the products of both loci in the expression of anthranilate synthetase is also clearly shown by these studies. Mutants of the *tryp-1Z* class accumulate wild-type levels of unassociated tryp-2 subunit but contain no discernible tryp-1 subunit which can either stimulate or inhibit the *in vitro* formation of anthranilate synthetase in the presence of added wild-type tryp-1 subunit. These mutants entirely lack anthranilate synthetase. The *tryp-1A* mutants have, in addition to a small pool of normal tryp-2 product, an abnormal 7S form of anthranilate synthetase. Our data reveal that this activity is due to the association of the wild type A_2 subunit with an altered *tryp-1* gene product which is a 3–4S component (probably I_2') to give an aberrant A_2I_2' 7S complex. The *tryp-1C* mutant which phenotypically mimics *tryp-2* mutants, is clearly the result of a mutation which, while preserving PRA isomerase and InGP synthetase, yields an I_4' which fails to generate the catalytic site for anthranilate synthetase although it can still associate with the A_2 subunit. Whether this failure is caused by the substitution of an amino acid essential to the active site or by a more general conformational change in the complex is not known. We conclude that, although the association of the two gene products is required for anthranilate synthetase activity, association is not sufficient. Mutations at both loci exist which result in subunits capable of association but incapable of producing anthranilate synthetase in the complex formed.

Mutants of the *tryp-1A*, *tryp-1C* and 10S *tryp-2* classes all produce detectable subunits. For this reason we feel that most of these strains are simple missense mutants. This conclusion is supported by the fact the majority of these strains revert to prototrophy with ultraviolet light (DEMOSS, JACKSON and CHALMERS 1967; CHALMERS 1968). The nonassociating *tryp-1Z* and *tryp-2* 7S mutants appear to represent a more extensive form of genetic damage, such as deletion, reading frame shift or nonsense mutation. Many strains of this type do not revert or else revert only at very low frequency with ultraviolet light (DEMOSS, JACKSON

TABLE 4

Properties and subunit structures of various altered complexes

Mutant	Isolated Gene Products* Activity, size†, inferred structure		State of the Multienzyme Complex: Activities				
	tryp-2	tryp-1	Anthranilate synthetase	PRA isomerase	InGP synthetase	Size	Inferred structure‡
tryp-2 7S	Enzymatically inactive, nonaggregating	Normal‖ 7S, (I_4)	0	+	+	7S	I_4
tryp-2 10S	Enzymatically inactive (A'_2)	Normal‖ 7S, (I_4)	0	+	+	10S	A'_2I_4
tryp-1A	Normal§ 4.5S (A_2)	Enzymatically inactive, abnormally aggregating, 3–4S (I'_2)	+	0	0	7S	$A_2I'_2$
tryp-1Z	Normal§ 4.5S (A_2)	Enzymatically inactive, nonaggregating	0	0	0	—	A_2
tryp-1C	Normal§ 4.5S (A_2)	Enzymatically active, abnormally aggregating 7S (I'_4)	0	+	+	10S	$A_2I'_4$

* Based on studies with double mutants
† Approximate sedimentation constant based on zone centrifugation in sucrose gradients
‡ The subunit designation of GAERTNER and DEMOSS (1969) is used. A = tryp-2 subunit, I = tryp-1 subunit, A′ and I′ = altered subunits).
‖ The normal *tryp-1* gene product has PRA isomerase and InGP synthetase activity.
§ The normal *tryp-2* gene product is enzymatically inactive in the nonaggregated form but has anthranilate synthetase activity in the aggregated form.

and CHALMERS 1967; CHALMERS 1968). Recently a number of these strains have been identified as nonsense mutants by their response to known nonsense suppressors.

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SUMMARY

A multienzyme complex of anthranilate synthetase, PRA isomerase and InGP synthetase is controlled by the *tryp-1* and *tryp-2* genes in Neurospora. Mutations at either locus lead to the formation of various types of altered complexes. Unassociated subunits of the complex were detected in extracts of some of the mutants by following the formation of the wild-type complex *in vitro* in the presence of an added excess of the other subunit. Double mutants were constructed which accumulated only the altered product of one of the two genes. These unassociated, altered subunits were identified and characterized by virtue of their interaction *in vitro* with wild-type subunits to form altered complexes with partial enzyme activities. From the results it was possible to infer the subunit structures of a number of the altered complexes. These studies show that the subunits of the multienzyme complex are the *tryp-1* and *tryp-2* gene products and that the specific association of these two types of subunits is necessary for the expression of anthranilate synthetase.

LITERATURE CITED

ARROYO-BEGOVICH, A. and J. A. DEMOSS, 1969 *In vitro* formation of an active multienzyme complex in the tryptophan pathway of *Neurospora crassa.* Proc. Natl. Acad. Sci. U.S. (in press).

CATCHESIDE, D. G., 1964 Interallelic complementation. Brookhaven Symp. Biol. **17**: 1–14.

CHALMERS, JR., H. J., 1968 The expression of an enzyme aggregate in *Neurospora.* Ph.D. thesis. University of California, La Jolla, Calif.

DEMOSS, J. A., 1965 The conversion of shikimic acid to anthranilic acid by extracts of *Neurospora crassa.* J. Biol. Chem. **240**: 1231–1235.

DEMOSS, J. A., R. W. JACKSON and J. H. CHALMERS, JR., 1967 Genetic control of the structure and function of an enzyme aggregate in the tryptophan pathway of *Neurospora crassa.* Genetics **56**: 413–424.

DEMOSS, J. A. and J. WEGMAN, 1965 An enzyme aggregate in the tryptophan pathway of *Neurospora crassa.* Proc. Natl. Acad. Sci. U.S. **54**: 241–247.

GAERTNER, F. and J. A. DEMOSS, 1969 Purification and Characterization of a multienzyme complex in the tryptophan pathway of *Neurospora crassa.* J. Biol. Chem. **244**: 2716–2725.

KAPLAN, S., Y. SUYAMA and D. M. BONNER, 1964 Fine structure analysis of the *td* locus of *Neurospora crassa.* Genetics **49**: 145–158.

LOWRY, O. H., N. ROSEBROUGH, S. L. FARR and R. J. RANDALL, 1964 Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**: 265–275.

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