BIOCHEMICAL, IMMUNOLOGICAL, AND GENETIC STUDIES WITH A NEW TYPE OF TRYPTOPHAN SYNTHETASE MUTANT OF *NEUROSPORA CRASSA*

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Previous studies have implicated indole-3-glycerol phosphate as an intermediate in the biosynthesis of tryptophan in *Escherichia coli* (Yanofsky, 1956*a*, *b*) and *Neurospora crassa* (Yanofsky and Rachmeler, 1958; DeMoss, Imai, and Bonner, 1958; Suskind and Jordan, 1959). It has also been established that this compound is converted to tryptophan by the enzyme tryptophan synthetase in both organisms (DeMoss and Bonner, 1959; Crawford and Yanofsky, 1958; Rachmeler, 1960). This enzyme system catalyzes three reactions:

$$\text{Indole} + \text{L-serine} \xrightarrow[\text{phosphate}]{\text{pyridoxal}} \text{L-tryptophan} \quad (1)$$

$$\text{Indoleglycerolphosphate} \rightleftharpoons \text{indole} + \text{triose phosphate} \quad (2)$$

$$\text{Indoleglycerolphosphate} + \text{L-serine} \xrightarrow[\text{phosphate}]{\text{pyridoxal}} \text{L-tryptophan} + \text{triose phosphate} \quad (3)$$

Of these, reaction 3 is believed to be the terminal reaction in tryptophan biosynthesis (Yanofsky and Rachmeler, 1958; Crawford and Yanofsky, 1958).

Tryptophan auxotrophs of *N. crassa* lacking the ability to convert indole to tryptophan (*td* mutants) have been studied by a number of investigators and have been found to fall into several distinguishable groups. Immunological tests have shown that some *td* mutants synthesize a protein which is related to the wild-type tryptophan synthetase, whereas others do not (Suskind, Yanofsky, and Bonner, 1955). These cross-reacting proteins are designated "CRM." Enzymatic tests have revealed that some cross-reacting proteins are active in one or more of the tryptophan synthetase reactions. Strains *td*2 and *td*71, for example, form cross-reacting proteins that are enzymatically active in reaction 2, but not in the other two reactions (DeMoss and Bonner, 1959; Suskind and Jordan, 1959). Strain *td*24 forms a cross-reacting protein that is enzymatically inactive in all of the reactions but can be activated to near wild-type activity by fractionation procedures which remove heavy metals (Suskind and Kurek, 1957).

Examinations of other tryptophan auxotrophs of *N. crassa* have uncovered still another type of cross-reacting protein. This protein is enzymatically active in reaction 1, but not in the other two reactions. The characteristics of this cross-reacting protein and the genetic relationships of the mutant strain from which it was obtained to other *td* mutants are the subjects of this report. Interactions of this new mutant type with various *td* alleles in heterocaryons will also be described.

MATERIALS AND METHODS

Of the various tryptophan auxotrophs of *N. crassa* used in this study, *td*1 and *td*16 do not form cross-reacting protein; *td*3, *td*6, and *td*7 form enzymatically inactive cross-reacting proteins; *td*2 forms a cross-reacting protein that is active in the conversion of indoleglycerolphosphate to indole; and strain *td*201[2] forms a cross-reacting protein that is active in the conversion of indole to tryptophan. All the *td* mutants mentioned, with the exception of *td*201, respond only to tryptophan. Strain *td*201 responds to indole as well as to tryptophan. The St. Lawrence wild-type strain, 74A, was used as the source of wild-type enzyme.

The strains studied were grown on minimal medium N (Vogel, 1956) containing 2% sucrose.

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[2] Strain *td*201 was originally designated A78 and was kindly supplied by M. Ahmad and D. G. Catcheside. Another tryptophan mutant, strain C-8, possessing the same enzymatic properties as *td*201 was isolated independently by R. Colburn at Stanford University.

The culture medium for the mutants was supplemented with 1 mg L-tryptophan/20 ml. After 60 to 72 hr of incubation, either in stationary Fernbach flasks at 25 C or with vigorous aeration in 20 liter carboys at approximately 30 C, the mycelium was harvested by filtration through cheesecloth, washed several times with distilled water, and lyophilized.

The method employed in the preparation of extracts and subsequent purification of the protein is a modification of procedures described by Yanofsky (1955) and Suskind (1957). The lyophilized mycelium was finely powdered with a mortar and pestle and extracted by adding sufficient cold 0.1 M potassium phosphate buffer, pH 7.8 (80 ml buffer/5 g dried mycelium), to wet the powder. After standing at 4 C for 30 min, the supension was centrifuged at 10,000 × *g* for 30 min. The supernatant solution was employed as the crude extract and was stored at −15 C.

All the succeeding steps were carried out at 4 C. A given volume of crude extract was adjusted to pH 7.0 with 0.1 N acetic acid and 1 ml protamine sulfate solution (15 mg protamine sulfate/ml 0.1 M phosphate buffer, pH 7.0) was added per 100 mg protein. After stirring for several minutes, the suspension was centrifuged and the precipitate discarded. The supernatant solution was adjusted to pH 5.8 with 1 N acetic acid and solid ammonium sulfate was added, with constant stirring, to 21% of saturation. After stirring for 20 min, the suspension was centrifuged and the precipitate taken up in 0.1 M phosphate buffer, pH 7.8. This solution, designated fraction I, was the fraction used in most experiments.

Further purification was occasionally carried out on a portion of this solution using calcium phosphate gel (Keilin and Hartree, 1938). The gel was added to the solution (5 mg gel/10 mg protein) and after 5 min the suspension was centrifuged and the sediment discarded. The supernatant solution (fraction II) was adjusted to pH 6.0, further fractionated with ammonium sulfate and the 15 to 30% fraction retained. This was designated fraction III. In all cases pyridoxal phosphate, 1.2×10^{-4} M, and glutathione, 10^{-3} M (both final concentrations), were added to the extracts and all solutions during purification. The protein content of extracts and purified preparations was determined by the method of Lowry et al. (1951).

The indole to tryptophan reaction was measured by following the disappearance of indole from a reaction mixture containing DL-serine, pyridoxal phosphate, and phosphate buffer at pH 7.8 (Yanofsky, 1955).

The indoleglycerolphosphate to tryptophan reaction was followed either by measuring indoleglycerolphosphate disappearance using the periodate oxidation method described by Yanofsky (1956*b*) or by assaying the tryptophan formed microbiologically (Yanofsky, 1954). In either case the reaction mixture contained 60 μmoles DL-serine, 0.16 μmole pyridoxal phosphate, 0.3 μmole indoleglycerolphosphate, and 200 μmoles potassium phosphate buffer at pH 7.8 in a final volume of 1 ml.

The indoleglycerolphosphate to indole reaction was measured by assaying the amount of indole formed (Yanofsky, 1955) in a reaction mixture containing 0.2 μmole indoleglycerolphosphate, 0.16 μmole pyridoxal phosphate, 100 μmoles potassium phosphate buffer at pH 7.8, 5 μmoles NH_2OH, and water in a final volume of 1.0 ml.

One unit of enzyme activity in each of the reactions mentioned is defined as the amount of enzyme required to catalyze the disappearance of 0.1 μmole of substrate or the appearance of 0.1 μmole of product in a 60-min incubation period at 37 C.

Cross-reacting protein was determined by the procedure of Suskind (1957) in both reactions 1 and 3. One unit of cross-reacting protein is defined as the amount of mutant protein which will protect one unit of wild-type enzyme activity from neutralization by antiserum. (The authors are indebted to Dr. S. R. Suskind for the antiserum.) Since the protective ability of cross-reacting proteins can often be measured in several of the reactions of tryptophan synthetase, cross-reacting protein (CRM) is designated $CRM_{In\ to\ Tryp}$, if determined in the indole to tryptophan reaction, and $CRM_{InGP\ to\ Tryp}$, if determined in the indoleglycerolphosphate to tryptophan reaction.

Michaelis constants were calculated from Lineweaver and Burk (1934) plots of kinetic data.

To obtain the mutant alleles in a compatible genetic background for heterocaryon formation (Garnjobst, 1953; Holloway, 1954), they were crossed in the same combinations which were subsequently to be tested for complementation: *td*201 by *td*1, *td*2, *td*3, *td*6, *td*7, and *td*16. The

crossing medium was that of Westergaard and Mitchell (1947) supplemented with indole (0.01 mg/ml) if *td*201 was the protoperithicial parent, or L-tryptophan (0.1 mg/ml) if one of the other *td* mutants was the protoperithicial parent. Conidia from one of the parents were placed on the crossing medium and grown for 6 to 8 days at 25 C. Conidia from the second parent were then sprinkled over the mycelium of the protoperithicial parent and the tubes incubated. Random spores from each cross were isolated, heat shocked, and grown in tryptophan supplemented media (Ryan, 1950). The various isolates were then tested nutritionally to determine whether they carried the *td*201 or the other *td* allele.

Heterocaryons were prepared by mixing 0.1 ml of heavy conidial suspensions (prepared from 5-day-old slants) of two strains in tubes containing 0.5 ml minimal medium plus 2% sucrose. The tubes were incubated at 25 C. After 3 to 5 days of incubation, the heterocaryons were transferred to and maintained on minimal agar slants. For extract preparation, the heterocaryons were grown in unsupplemented liquid minimal medium.

RESULTS

Tryptophan synthetase of strain td201. Tryptophan auxotroph *td*201 differs from all the tryptophan synthetase mutants of Neurospora previously examined in that it can use indole to satisfy its tryptophan requirement. If this property was indicative of another type of alteration of tryptophan synthetase, it might be expected that the mutationally altered site in this strain would be within the *td* locus. To examine this possibility, 1,100 random ascospores were isolated from crosses between strain *td*201 and *td*2. In addition, several hundred random spores from crosses of *td*201 by *td*1, *td*3, *td*7, and *td*16 were also tested. In no case was any tryptophan independent ascospore recovered, indicating that the mutation characteristic of strain *td*201 is at or very near the site of the mutational alteration in the other *td* mutants examined. Approximately equal numbers of parental types were recovered from these crosses. Similar evidence of allelism of *td*201 and other *td* mutants has recently been reported by Ahmad and Catcheside (1960).

Extracts of strain *td*201 were examined for enzymatic activity in the three reactions catalyzed by wild-type tryptophan synthetase.

TABLE 1

*Assays for three activities of tryptophan synthetase with extracts of wild type and strain td201 of Neurospora crassa**

Extract	Reaction 1 (Indole Used: hr: mg Protein)	Reaction 2 (Indole Formed/hr)	Reaction 3 (Tryptophan Formed/hr)
	μmoles	*μmole*	*μmoles*
Wild-type, fraction I	1.85	0.028	1.03
*td*201, fraction I	13.78	0.0	0.0

* *See* Materials and Methods for fraction preparation, reaction mixtures, and assay conditions.

TABLE 2

Purification of the tryptophan synthetase protein of strain td201

Preparation*	Specific Activity†	Recovery
	units/mg protein	%
Crude extract	10.8	
Fraction I	137.8	74
Fraction II	301.5	67
Fraction III	462.2	36

* *See* Materials and Methods for fractionation procedure and assay conditions.

† Reaction 1.

These assays revealed that *td*201 forms a protein that is active in the conversion of indole to tryptophan (reaction 1), but is inactive in the other two reactions (Table 1). This activity accounts for the growth behavior of this strain.

The purification procedure which had been effective with wild-type tryptophan synthetase (*see* Materials and Methods) was used to purify *td*201 cross-reacting protein. Approximately a 45-fold purification was achieved (Table 2) and the *td*201 enzyme invariably fractionated like the wild-type enzyme. It is of interest to note that, in general, crude extracts of strain *td*201 are three times as active in reaction 1 as crude extracts of the wild-type strain. Several tryptophan auxotrophs blocked earlier in the tryptophan pathway also form somewhat elevated levels of tryptophan synthetase under certain growth conditions.

The stability of the mutant and normal enzymes during various treatments was also

TABLE 3

Relative stabilities of td201 and wild-type tryptophan synthetase

Treatment	Inactivation, Indole to Tryptophan Activity	
	Wild type, fraction I	*td*201, fraction I
	%	%
Dialysis for 2 hr at 4 C against 0.1 M phosphate buffer at pH 7.8	40	46
Heat at 60 C for 5 min	95	58
Addition of 1 N acetic acid to pH 4.5 and neutralize	80	78
Storage for 20 hr at 4 C	21	10

TABLE 4

*Comparison of Michaelis constants for indole and serine obtained with td201 and wild-type tryptophan synthetase**

Extract	K_m Determination for	K_m
		M
*td*201, fraction I	Serine	2.9×10^{-3}
*td*201, fraction I	Indole	1.8×10^{-5}
Wild-type, fraction I	Serine	4.1×10^{-3}
Wild-type, fraction I	Indole	2.2×10^{-5}

* The final concentrations of pyridoxal phosphate and pH 7.8 phosphate buffer present in all the experiments were 1×10^{-4} M and 0.1 M, respectively. The K_m for serine was determined in the presence of an excess of indole (4×10^{-4} M) and the K_m for indole in the presence of an excess of serine (5×10^{-2} M).

examined (Table 3). It can be seen that the normal and mutant enzymes differ with respect to inactivation by heat at 60 C. A comparison of affinities (Table 4) indicated that both enzymes had a similar affinity for indole, whereas the mutant enzyme appeared to have a slightly higher affinity for serine.

The neutralizing effect of rabbit antitryptophan-synthetase serum on both enzymes was compared. As can be seen in Fig. 1, the indole to tryptophan activity of both enzymes was equally sensitive to antibody, demonstrating that in the indole to tryptophan reaction the mutant enzyme is as active per unit antigenic material as the wild-type enzyme.

Although antibody inhibition of indoleglycerol-

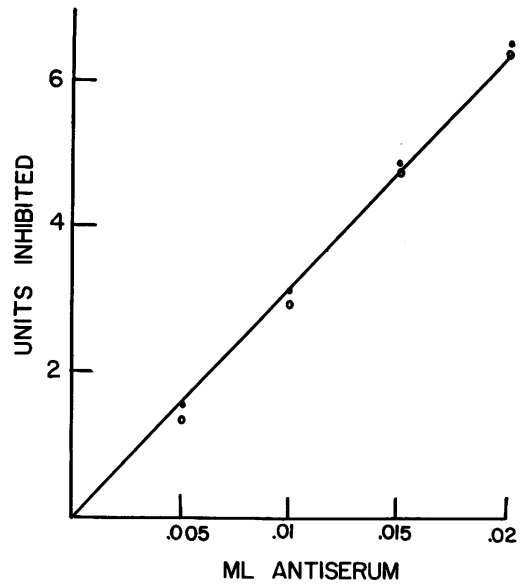

Fig. 1. Neutralization of indole to tryptophan activity by antitryptophan synthetase serum. The enzyme (4 to 8 units) and antiserum were mixed in a solution containing 0.2 ml 0.5 M phosphate buffer at pH 7.8 and 0.1 ml 0.5% bovine plasma albumin. After 15 min at 4 C, the substrates for the indole to tryptophan assay were added and the mixture was further incubated for 60 min at 37C. The reaction mixture was assayed for indole. O——O = wild-type tryptophan synthetase, ●——● = *td*201 tryptophan synthetase.

phosphate to tryptophan activity obviously could not be determined with *td*201 preparations, it was possible to measure the *td*201 enzyme as cross-reacting protein in this reaction. If a single protein were responsible for both the immunological and the enzymatic activity of *td*201 extracts, then the ratio of $CRM_{InGP\ to\ Tryp}$ to enzymatic activity in the indole to tryptophan reaction should be approximately the same as the ratio of the two relative reaction rates (of these reactions) with the wild-type enzyme (Table 1). The amount of $CRM_{InGP\ to\ Tryp}$ was determined and a comparison made with the indole to tryptophan activity of the same preparation. These studies showed that the ratio of the two (1:1.58) was approximately the same as the ratio of the two enzymatic activities of the wild-type enzyme (1:1.92). This finding further supports the conclusion that the *td*201 protein is an altered tryptophan synthetase which is fully active immunologically but enzymatically

active only in one of the tryptophan synthetase reactions.

Heterocaryon studies. Since the altered tryptophan synthetases produced by some of the *td* mutants possessed different enzymatic properties, it was possible to examine extracts of heterocaryons for the products normally produced by the participating mutants and for any "wild-type-like" tryptophan synthetase activity formed as a result of complementation. The various combinations of mutants tested for heterocaryon formation are listed in Table 5. A negative result indicates that there were no tryptophan independent heterocaryons in 10 mixtures—each mixture containing a different isolate of each type from the same cross. The absence of a tryptophan independent heterocaryon in these tests does not necessarily mean that the two mutant alleles do not complement since the isolates used were obtained from a single cross and compatibility differences might still exist. Compatibility tests with a tester stock were performed, however, and the results indicated that there should have been some compatible combinations in each series of mixtures. Studies with more isogenic stocks of several of these mutants have shown that they can complement (Lacy and Bonner, 1958). Of importance from the data presented here is that *td*201 readily complemented with all of the formers of cross-reacting protein examined (strains *td*2, *td*3, *td*6, and *td*7) but failed to complement with the two strains which do not form cross-reacting protein (strains *td*1 and *td*16).

TABLE 5

*Heterocaryon tests with various td mutants**

Partner 2	Partner 1						
	*td*201	*td*1	*td*2	*td*3	*td*6	*td*7	*td*16
*td*201	−	−	+	+	+	+	−
*td*1		−	−	−	−	−	−
*td*2			−	−	−	−	−
*td*3				−	+	−	−
*td*6					−	−	−
*td*7						−	−
*td*16							−

* Tests were performed by mixing 0.1-ml aliquots of conidial suspensions of the two strains in 0.5 ml minimal medium and incubating at 25 C for up to 6 days. (+) = Positive heterocaryon; (−) = no growth after 6 days.

Significance of indoleglycerolphosphate to tryptophan reaction in enzyme assays with extracts of heterocaryons. The mutants examined which formed tryptophan independent heterocaryons normally produce either inactive enzyme (*td*3, *td*7) or partially active enzyme (*td*2, *td*201), e.g., enzyme which catalyzes only one of the three reactions catalyzed by wild-type tryptophan synthetase. Since extracts of the mutants studied cannot carry out the indoleglycerolphosphate to tryptophan reaction, this reaction becomes a measure of wild-type-like activity. As shown in Table 6, extracts of strains *td*2 and *td*201, each active in a different reaction, when mixed failed to yield a level of indoleglycerolphosphate to tryptophan activity significantly above the indoleglycerolphosphate to indole activity of the *td*2 preparation itself. With wild-type tryptophan synthetase, the relative reaction rates for the indoleglycerolphosphate to tryptophan and indoleglycerolphosphate to indole reactions are approximately 1:0.03 (Table 1). Therefore, in an examination of heterocaryon extracts any indoleglycerolphosphate to tryptophan activity that is substantially in excess of the indoleglycerolphosphate to indole activity would presumably represent the restoration of wild-type-like activity. Through the use of the ratios of the relative reaction rates of the three reactions of wild-type tryptophan synthetase it is possible to estimate the amounts of indole to tryptophan and indoleglycerolphosphate to indole activities present which can be attributed to a restored wild-type-like protein. In addition, immunological techniques could be used to quantitate the amount of cross-reacting protein.

Examination of an extract of a td210-td3 heterocaryon. The results obtained in an enzymatic and immunological examination of an extract of a heterocaryon formed between strains *td*201 and *td*3 are represented in Table 7. From the experimental results obtained and the calculations described in the table, *td*201 activity (indole to tryptophan activity), cross-reacting protein (enzymatically inactive, immunologically active protein like cross-reacting protein of *td*3) and wild-type activity (indoleglycerolphosphate to tryptophan activity) all were found in the extracts. Assuming each of these represents a different tryptophan synthetase protein they would be present in the ratio 4:7:1, in the order given. It is important to note that the experimentally determined values for the indoleglycerolphosphate

TABLE 6

Enzyme assays of mixtures of extracts of td mutants

Fraction I Extract	Relative Reaction Rates		
	Reaction 1	Reaction 2	Reaction 3
Wild-type	100*	1.5	55
*td*201	100	0.0	0.0
*td*2	0	1.3	0.0
*td*3	0	0.0	0.0
*td*7	0	0.0	0.0
*td*2 + *td*201	100	1.3	2.0
*td*3 + *td*201	100	0.0	0.0
*td*7 + *td*201	100	0.0	0.0

* The values for reaction 1 arbitrarily set at 100.

TABLE 7

Analysis of an extract of a td3-td201 heterocaryon

Activity*	Observed	Calculated
	units/ml	*units/ml*
(*a*) Indole → tryptophan	246	
(*b*) Indoleglycerolphosphate → tryptophan	25	
(*c*) Indoleglycerolphosphate → indole	0.73	
(*d*) Indole → tryptophan (attributed to wild type)		50
(*e*) Indole → tryptophan (attributed to *td*201)		196
(*f*) $CRM_{In\ to\ Tryp}$	332	

* (*d*) Derived by multiplying line (*b*) × 2. (*e*) Derived by subtracting line (*d*) from line (*a*).

to tryptophan and indoleglycerolphosphate to indole activities were in a ratio of 33:1—the same ratio usually observed for these activities with the wild-type enzyme (Table 1). Thus the heterocaryon appears to form some enzyme with relative reaction rates equivalent to those of the wild-type protein.

Examination of an extract of a td201-td7 heterocaryon. In this heterocaryon as in the previous one it appears that at least three proteins are formed: *td*201 tryptophan synthetase, cross-reacting protein (*td*7(?)), and a wild-type-like protein (Table 8). The ratio of these three activities in the order mentioned is 16:12:1. In comparison with the total amount of tryptophan synthetase protein formed there is much less wild-type-like activity than in the *td*3-*td*201 heterocaryon. Here the ratio of experimentally determined indoleglycerolphosphate to tryptophan and indoleglycerolphosphate to indole activities is 41:1, again approximating that of the ratio obtained with the wild-type enzyme.

Examination of an extract of a td201-td2 heterocaryon. The analysis of the products formed by this heterocaryon (Table 9) proved to be somewhat more complicated, although perhaps more informative, since both complementing partners formed enzymatically active cross-reacting protein. The amount of *td*2 protein was estimated by subtracting from the experimentally determined indoleglycerolphosphate to indole activity that portion which could be attributed to wild-type activity (determined by using the ratio of indoleglycerolphosphate to tryptophan and indoleglycerolphosphate to indole activity of wild-type tryptophan synthetase of 1:0.03). In previous experiments with *td*2 cross-reacting protein it was shown that 1 unit of indoleglycerolphosphate to indole activity was equivalent to 80 units of $CRM_{In\ to\ Tryp}$ (Rachmeler, 1960). Using this value, the fraction of the cross-reacting protein found in the heterocaryon which can be attributed to the *td*2 mutant protein can be calculated (Table 9). It can be seen (line *j*) that more $CRM_{In\ to\ Tryp}$ was detected in the extract of the heterocaryon than could be accounted for by the cross-reacting protein attributed to the *td*2 mutant. This excess cross-reacting protein may represent an enzymatically inactive, immunologically active, fourth product formed by this heterocaryon. Assuming that the activities detected and calculated are at-

TABLE 8

Analysis of an extract of a td7-td201 heterocaryon

Activity*	Observed	Calculated
	units/ml	*units/ml*
(*a*) Indole → tryptophan	296	
(*b*) Indoleglycerolphosphate → tryptophan	8.6	
(*c*) Indoleglycerolphosphate → indole	2.1	
(*d*) Indole → tryptophan (attributed to wild type)		17
(*e*) Indole → tryptophan (attributed to *td*201)		279
(*f*) $CRM_{In\ to\ Tryp}$	184	

* (*d*) Derived by multiplying line (*b*) × 2. (*e*) Derived by subtracting line (*d*) from line (*a*).

TABLE 9

Analysis of an extract of a td2-td201 heterocaryon

Activity*	Observed	Calculated
	units/ml	*units/ml*
(*a*) Indole → tryptophan	285	
(*b*) Indoleglycerolphosphate → tryptophan	48	
(*c*) Indoleglycerolphosphate → indole	2.3	
(*d*) Indole → tryptophan (attributed to wild type)		96
(*e*) Indole → tryptophan (attributed to *td*201)		189
(*f*) Indoleglycerolphosphate → indole (attributed to wild type)		1.4
(*g*) Indoleglycerolphosphate → indole (attributed to *td*2)		0.9
(*h*) $CRM_{In\ to\ Tryp}$	121	
(*i*) $CRM_{In\ to\ Tryp}$ (attributed to *td*2)		72
(*j*) Excess $CRM_{In\ to\ Tryp}$		49

* (*d*) Derived by multiplying line (*b*) × 2. (*e*) Derived by subtracting line (*d*) from line (*a*). (*f*) Derived by multiplying line (*b*) × 0.03. (*g*) Derived by subtracting line (*f*) from line (*c*). (*i*) Derived by multiplying line (*g*) × 80. (*j*) Derived by subtracting line (*i*) from line (*h*).

tributable to four tryptophan synthetase protein species, *td*201 protein, *td*2 protein, wild-type protein, and an enzymatically inactive protein, these would be present in the ratio 2:0.75:1:0.51. However, it should be mentioned that indoleglycerolphosphate to indole activity is difficult to quantitate with low levels of activity and thus the fourth product may not exist. Examinations of separate batches of mycelium from *td*201-*td*2 heterocaryons have, however, given essentially similar results.

DISCUSSION

The formation of altered proteins as a result of mutation has been demonstrated in investigations with several gene-protein systems (for reviews *see* Fincham (1960) and Yanofsky and St. Lawrence (1960)). In studies with tryptophan synthetase, in both *E. coli* and *N. crassa*, mutations have been shown to lead to the formation of many distinguishable altered proteins (Suskind et al., 1955; Yanofsky, 1960). In *N. crassa* at least five major classes of tryptophan synthetase mutants have now been recognized:

Class 1 contains mutants which do not form cross-reacting protein or an enzymatically active protein, e.g., *td*1, *td*16 (Suskind et al., 1955; Rachmeler, 1960).

Class 2 mutants form cross-reacting protein but the protein is enzymatically inactive in all three reactions of tryptophan synthetase, e.g., *td*3, *td*7 (Suskind et al., 1955; Rachmeler, 1960).

Class 3 contains one mutant, strain *td*24, which forms an altered enzyme that is unusually sensitive to heavy metals (Suskind and Kurek, 1957).

Class 4 mutants form a cross-reacting protein which is enzymatically active only in the conversion of indoleglycerolphosphate to indole, e.g., *td*2, *td*71 (DeMoss and Bonner, 1959; Suskind and Jordan, 1959).

Finally, in class 5 there are mutants such as *td*201 which form a cross-reacting protein which is active only in the conversion of indole to tryptophan.

Further studies have provided evidence indicating that there are subclasses in the major classes of *td* mutants mentioned (DeMoss and Bonner, 1959; Rachmeler, 1960). Strains *td*2 and *td*71, both of which fall into class 4, differ from each other in their requirements for enzymatic activity. Both strains require pyridoxal phosphate for the conversion of indoleglycerolphosphate to indole, but *td*71 requires serine in addition for maximal activity (DeMoss and Bonner, 1959). A note of further interest is that although pyridoxal phosphate is required for maximal activity by the cross-reacting protein of *td*2, the presence of hydroxylamine does not affect its activity (Rachmeler, 1960). Hydroxylamine completely inhibits the pyridoxal phosphate requiring reactions of wild-type tryptophan synthetase. These facts suggest that pyridoxal phosphate may be required for a structural modification of the enzyme surface of the cross-reacting protein of *td*2 rather than participating as a coenzyme in the enzymatic reaction. A similar conclusion has been drawn for the cross-reacting protein of strain *td*71 on the basis of the serine requirement of this protein (DeMoss and Bonner, 1959). In view of the behavior of the protein of *td*2 it would be of interest to determine the effect of hydroxylamine on the activity of *td*71 protein which also requires

pyridoxal phosphate for activity. Undoubtedly other types of altered tryptophan synthetase proteins will be detected as more varied methods are applied in investigations with cross-reacting proteins.

The unique properties of the cross-reacting proteins formed by many of the *td* alleles have been exploited in a preliminary study of complementation in heterocaryons. The findings reported here demonstrate that certain *td* alleles can form tryptophan independent heterocaryons and that extracts of these heterocaryons exhibit tryptophan synthetase activity in the indoleglycerolphosphate to tryptophan reaction. Whether this activity is associated with a wild-type protein or is the result of the interaction of two mutant proteins was not determined. Thus the data obtained cannot be used in support of any of the proposed mechanisms of complementation (Catcheside and Overton, 1958; Giles, Partridge, and Nelson, 1957; Fincham, 1959). The results do indicate, however, that the nuclei of allelic mutants, in addition to whatever contribution they make to the reconstituted wild-type activity, probably continue to direct the production of their characteristic altered proteins. In addition, there is a suggestion in one case that a fourth tryptophan synthetase protein may be formed. Before this fourth product or the wild-type-like protein can be established as a separate protein species, it will be necessary to develop techniques which will permit the separation of the different tryptophan synthetase proteins. The use of cross-reacting proteins with different activities or distinguishable properties such as those employed in the present study will undoubtedly be of great help in the further study of complementation.

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SUMMARY

The properties of a new type of *td* mutant of *Neurospora crassa* have been studied. Unlike the *td* mutants previously examined, this strain, *td*201, can use indole to satisfy its tryptophan requirement. The site of the mutation responsible for the block in tryptophan synthesis in strain *td*201 has been shown to be at or very close to the *td* locus. Immunological tests have shown that strain *td*201 produces a protein which cross reacts with antitryptophan-synthetase serum. Enzymatic analyses demonstrated that this *td*201 protein is active in only one of the three reactions of wild-type tryptophan synthetase—the conversion of indole to tryptophan. In this reaction the mutant protein is as active per unit of antigenic material as the wild-type enzyme. The mutant enzyme does, however, show several differences from the normal enzyme.

Strain *td*201 formed tryptophan-independent heterocaryons with all the cross-reacting protein (CRM)-forming *td* mutants. Analyses of extracts of several of these heterocaryons revealed the presence of wild-type-like activity in addition to the altered products normally formed by the participating mutants. In one heterocaryon, *td*201-*td*2, the data obtained suggest that a fourth product may be formed. These studies demonstrate the applicability of tryptophan synthetase mutants to the study of the phenomenon of complementation.

REFERENCES

AHMAD, M., AND D. G. CATCHESIDE 1960 Physiological diversity amongst tryptophan mutants in *Neurospora crassa*. Heredity, **15**, 55–64.

CATCHESIDE, D. G., AND A. OVERTON 1958 Complementation between alleles in heterocaryons. Cold Spring Harbor Symposia Quant. Biol., **23**, 137–140.

CRAWFORD, I. P., AND C. YANOFSKY 1958 On the separation of the tryptophan synthetase of *Escherichia coli* into two protein components. Proc. Natl. Acad. Sci. U. S., **44**, 1161–1170.

DEMOSS, J. A., AND D. M. BONNER 1959 Studies on normal and genetically altered tryptophan synthetase from *Neurospora crassa*. Proc. Natl. Acad. Sci. U. S., **45**, 1405–1412.

DEMOSS, J. A., M. IMAI, AND D. M. BONNER 1958 Studies on tryptophan biosynthesis in *Neurospora crassa*. Bacteriol. Proc., **1958**, 112.

FINCHAM, J. R. S. 1959 On the nature of the glutamic dehydrogenase produced by inter-allele complementation at the *am* locus of *Neurospora crassa*. J. Gen. Microbiol., **21**, 600–611.

FINCHAM, J. R. S. 1960 Genetically controlled differences in enzyme activity. Advances in Enzymol., **22**, 1–43.

GARNJOBST, L. 1953 Genetic control of heterocaryosis in *Neurospora crassa*. Am. J. Botany, **40**, 607–614.

GILES, N. H., C. W. H. PARTRIDGE, AND N. J.

NELSON 1957 The genetic control of adenylossuccinase in *Neurospora crassa*. Proc. Natl. Acad. Sci. U. S., **43**, 305–317.

HOLLOWAY, B. W. 1954 Genetic control of heterocaryosis in *Neurospora crassa*. Genetics, **40**, 117–129.

KEILIN, D., AND E. F. HARTREE 1938 Mechanism of the decomposition of hydrogen peroxide by catalase. Proc. Roy. Soc. (London), Ser. B, **124**, 397–405.

LACY, A. M., AND D. M. BONNER 1958 Complementarity between alleles at the *td* locus in *Neurospora crassa*. Proc. Intern. Congr. Genet., 10th Congr. (Montreal), **2**, 157.

LINEWEAVER, H., AND D. BURK 1934 The determination of enzyme dissociation constants. J. Am. Chem. Soc., **56**, 658–666.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL 1951 Protein measurement with the Folin-phenol reagent. J. Biol. Chem., **193**, 265–275.

RACHMELER, M. 1960 A study of the normal and mutationally altered forms of tryptophan synthetase of *Neurospora*. Ph.D. Thesis, Western Reserve University, Cleveland.

RACHMELER, M., AND C. YANOFSKY 1959 Biochemical and genetic studies with a new *td* mutant type in *Neurospora crassa*. Bacteriol. Proc., **1959**, 30.

RYAN, F. J. 1950 Selected methods of Neurospora genetics. In *Methods in medical research*, vol. 3, pp. 51–75. Edited by R. W. Gerard. The Year Book Publishers, Inc., Chicago.

SUSKIND, S. R. 1957 Properties of a protein antigenically related to tryptophan synthetase in *Neurospora crassa*. J. Bacteriol., **74**, 308–318.

SUSKIND, S. R., AND E. JORDAN 1959 Enzymatic activity of a genetically altered tryptophan synthetase of *Neurospora crassa*. Science, **129**, 1614–1615.

SUSKIND, S. R., AND L. I. KUREK 1957 Enzyme-inhibitor complex in a tryptophan-requiring mutant of *Neurospora crassa*. Science, **126**, 1068–1069.

SUSKIND, S. R., C. YANOFSKY, AND D. M. BONNER 1955 Allelic strains of *Neurospora* lacking tryptophan synthetase. A preliminary immunochemical characterization. Proc. Natl. Acad. Sci. U. S., **41**, 577–582.

VOGEL, H. J. 1956 A convenient growth medium for *Neurospora* (medium N). Microbial Genetics Bull., **13**, 42–43.

WESTERGAARD, M., AND H. K. MITCHELL 1947 *Neurospora*. V. A synthetic medium favoring sexual reproduction. Am. J. Botany, **34**, 573–578.

YANOFSKY, C. 1954 The absence of a tryptophan-niacin relationship in *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol., **68**, 577–584.

YANOFSKY, C. 1955 Tryptophan synthetase from *Neurospora*. In *Methods in enzymology*, vol. 2, pp. 233–238. Edited by S. P. Colowick and N. O. Kaplan. Academic Press, Inc., New York.

YANOFSKY, C. 1956*a* Indole-3-glycerol phosphate, an intermediate in the biosynthesis of indole. Biochim. et Biophys. Acta, **20**, 438–439.

YANOFSKY, C. 1956*b* The enzymatic conversion of anthranilic acid to indole. J. Biol. Chem. **223**, 171-184

YANOFSKY, C. 1960 The tryptophan synthetase, system. Bacteriol. Rev., **24**, 221–245.

YANOFSKY, C., AND M. RACHMELER 1958 The exclusion of free indole as an intermediate in the biosynthesis of tryptophan in *Neurospora crassa*. Biochim. et Biophys. Acta, **28**, 640–641.

YANOFSKY, C., AND P. ST. LAWRENCE 1960 Gene action. Ann. Rev. Microbiol., **14**, 311-340.