*STUDIES ON NORMAL AND GENETICALLY ALTERED TRYPTOPHAN SYNTHETASE FROM NEUROSPORA CRASSA**

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Recent experiments have implicated indole-3-glycerol phosphate (InGP) as an intermediate in the conversion of anthranilic acid to tryptophan in *Escherichia coli*[1] and *Neurospora crassa*.[2, 3] In addition, Yanofsky[4] has demonstrated that a single enzyme, trytophan synthetase, from *E. coli* catalyzes the following three reactions:

$$\text{indole} + \text{serine} \rightarrow \text{tryptophan} \quad (1)$$

$$\text{InGP} + \text{serine} \rightarrow \text{tryptophan} + \text{triose phosphate} \quad (2)$$

$$\text{InGP} \rightleftharpoons \text{indole} + \text{triose phosphate} \quad (3)$$

Reaction (1) has been studied in some detail in extracts from *Neurospora crassa*.[5] This reaction requires the presence of pyridoxal phosphate. Further studies demonstrated that extracts from wild type *N. crassa* also catalyze reactions (2) and (3), and suggested that InGP is converted to tryptophan by wild type *N. crassa* without the formation of free indole.[2, 3]

While a majority of *N. crassa* auxotrophs which lack tryptophan synthetase (td mutants) accumulate indole glycerol in their growth filtrate, two accumulate indole.[6] If the final step in tryptophan biosynthesis is the conversion of InGP to tryptophan, the accumulation of indole by these td mutants cannot be explained by the absence of a single enzyme.

The following data indicate that the accumulation of indole by td mutants is attributable to the presence of an altered tryptophan synthetase, which catalyzes reaction (3) but not reactions (1) or (2).

Materials and Methods.—Mutant td 2, which was previously studied,[7] and td 71, which was obtained by X-irradiation of the wild type strain 74A,[6] were used. Both of these mutants require tryptophan for growth and accumulate indole in their culture filtrates, while all other td mutants which have been isolated accumulate indole glycerol in their growth medium. Both td 2 and td 71 have been shown to be allelic with other td mutants.[6]

Wild type 74A, which has been used for these studies, was grown on minimal medium[8] and mutant strains td 2 and td 71 were grown on minimal medium plus 100 μg L-tryptophan per ml. Cultures were grown on a rotary shaker at 30°C for 48–72 hours. Mycelia were filtered through cheesecloth, washed and lyophilized. The lyophilized mycelia were powdered in a Wiley mill and stored at −15°C.

Extracts were prepared at 4°C by grinding one part powdered, lyophilized mycelia with two parts alumina A-301 with the gradual addition of 15 parts of 0.05 *M* phosphate buffer, pH 7.8. The extract was centrifuged at 100,000 × *g* for 30 min and the supernatant used as the crude extract. The crude extract always contained 15–20 mg of protein per ml.

Fractionation procedures were carried out for either concentration or purification of enzyme activity and were the same for both wild type and mutant strains. To

concentrate the activity in crude extracts, 14 ml of 1.5 per cent protamine sulfate was added to 100 ml of extract. The mixture was stirred for 10 min and then centrifuged at 20,000 × *g*. Solid ammonium sulfate was added to the supernatant to a concentration of 65 per cent saturation. The precipitate formed was collected by centrifugation at 20,000 × *g* for 15 min and dissolved in 10–20 ml of 0.05 *M* phosphate buffer, pH 7.8. This preparation was used as the concentrated extract.

Purification of activity was carried out as follows: After protamine sulfate treatment, the supernatant was brought to 50 per cent saturation with solid ammonium sulfate, mixed for 10 min and centrifuged at 20,000 × *g* for 15 min. The precipitate was dissolved in 10.0 ml of 0.025 *M* phosphate buffer, pH 7.8 (Step I). This preparation was diluted with 20.0 ml of 0.05 *M* phosphate buffer, pH 7.0, and mixed with 9.0 ml of alumina C γ gel, pH 7.0, for 10 min. The mixture was centrifuged at 20,000 × *g* for 10 min and the precipitate discarded (Step II). The supernatant was then fractionated with a saturated solution of ammonium sulfate. The precipitate which formed between 25 and 30 per cent saturation with ammonium sulfate was collected by centrifugation and dissolved in 3.0 ml of 0.05 *M* phosphate buffer, pH 7.8 (Step III). This fraction was designated fractionated extract and usually exhibited a 45–50 fold increase in specific activity over the crude extract with about 25–30 per cent recovery. All the fractionation operations were carried out at 0–5°C.

Reaction (1) was measured by the disappearance of indole.[9] Reaction (2) was determined by the appearance of tryptophan by a modification of a procedure by Kupfer.[10] Assays for reaction (1) and (2) in mutant extracts were set up to detect at least 1 per cent of the wild type activity level. Reaction (3) was assayed by indole formation using the same procedure employed in the measurement of reaction (1). The reverse reaction, InGP formation from indole, was measured by the periodate method of Yanofsky.[1] CRM (cross reacting material) was determined according to the procedure of Suskind.[11] Protein was assayed with the Folin reagent.[12]

Results.—Activities of wild type tryptophan synthetase:—Early studies provided relatively convincing evidence that indole was an intermediate product in tryptophan biosynthesis and was the substrate for tryptophan synthetase.[13, 9] However, the demonstration that extracts from *Neurospora* are capable of catalyzing reactions (1), (2), and (3), and the evidence for the participation of a single enzyme in these three reactions in *E. coli* casts some doubt on the previous interpretations. An attempt was made, therefore, to establish whether these reactions were catalyzed by one, or more than one enzyme in *Neurospora*. Table 1 shows the data obtained when the three activities (reaction (1), (2), and (3)) were followed over a 34-fold purification range of the wild type enzyme. The ratio of the three activities remains essentially constant within the limits of the assay procedures employed. Further, it was found that the three activities are eluted from a DEAE cellulose ion exchange column in the same fractions. These results indicate that a single protein, tryptophan synthetase, is responsible for reactions (1), (2), and (3).

It may be concluded that tryptophan synthetase is responsible for the formation of indole as well as tryptophan. The absence of tryptophan synthetase from td 2 and td 71 extracts, as judged by the absence of reaction (1), and the accumulation of indole by these mutants would therefore appear to be contradictory.

TABLE 1

FRACTIONATION OF WILD TYPE ACTIVITIES*

Fraction	Reaction (2) μM/hr/mg pr	Ratio reaction (1)/-reaction (2)	Ratio reaction (3)/-reaction (2)
Crude extract	0.055	1.8	0.016
Step I*	0.172	1.5	0.014
Step II	0.209	1.7	...
Step III	1.89	1.3	0.020

* See *Methods* for fractions steps.
† For assay of reaction (2), the complete system contained 25 μM phosphate buffer, pH 7.8, 20 μg B_6P, 0.1 μM InGP, 80 μM DL-serine and, 0.05 μM glutathione in a final column of 1.0 ml. The complete system for the assay of reaction (3) is the same as in Table 4. The complete system for the assay of reaction (1) is that of Yanofsky.[9]

Activities of indole accumulating mutants:—Crude extracts from td 2 and td 71 as well as 74A were examined for their ability to convert InGP to indole. When the extracts were properly supplemented (see below), the reaction was found in td 2 and td 71 as well as wild type, but not in td 6 which is an indole glycerol accumulating td mutant (Table 2). Reaction (3) could be detected in td 2 extracts only when the

TABLE 2

RELATIVE LEVEL OF REACTION (3) IN VARIOUS CRUDE EXTRACTS UNDER OPTIMAL CONDITIONS

Extract	μM/hr/mg pr*
74A (Wild type)	0.0011
td 2	0.0030
td 71	0.040
td 6	0.0000

* The assay system for each strain is the same as the complete systems shown in Table 3. The td 6 extract was assayed with the complete system used for td 71 extract.

extracts were concentrated by ammonium sulfate precipitation so that large quantities of protein could be used. This procedure also had to be used routinely for the detection of reaction (3) activity in wild type extracts. The specific activities obtained with td 2 extracts were of the same order of magnitude as the reaction in crude extracts of wild type. In td 71, on the other hand, a relatively high level of activity was obtained. Reaction (1) and (2) could not be demonstrated in crude or purified preparations of extract from td 2 and td 71. It appeared, therefore, that these mutants had lost the ability to catalyze two of the reactions catalyzed by wild type tryptophan synthetase, but still retained the third, i.e., InGP → indole.

Data demonstrating the requirements for the conversion of InGP to indole by extracts from the three strains in Table 3. Surprisingly, in each case a different set of requirements was found. In the case of wild type extract, the reaction was always measured in the absence of serine to avoid the complication of simultaneous tryptophan formation. It can be seen, however, that pyridoxal phosphate is not

TABLE 3

REQUIREMENTS FOR CONVERSION OF InGP TO INDOLE

	μM/hr/mg protein		
	74A*	td 2†	td 71‡
Complete§	0.017	0.003	0.040
minus InGP	0.000	0.000	0.001
minus B_6P	0.032	0.001	0.007
minus Serine	...	0.003	0.007

* 74A—fractionated extract (see *Methods*).
† Td 2—concentrated extract (see *Methods*).
‡ Td 71—crude extract.
§ The complete system for 74A contained 25 μM phosphate buffer, pH 7.8, 20 μg B_6P and 0.2 μM InGP in a final volume of 1.0 ml. For td 2 and td 71, 80 μM DL-serine was added. The incubation was for 1 hr at 37°C.

required by the wild type and is, in fact, somewhat inhibitory. In td 2 extracts, the reaction requires pyridoxal phosphate for maximum indole formation, but serine does not stimulate the reaction. The reaction in td 71 extracts requires both serine and pyridoxal phosphate for the maximum rate in the crude extracts and an absolute requirement for each could be demonstrated in more purified extracts.

The reverse reaction, indole + triose phosphate → InGP, could be demonstrated with each preparation if, in addition to indole, hexose diphosphate and aldolase were added as a source of triosephosphate. For each strain the requirements were the same as for the conversion of InGP to indole.

Relationship of activities to tryptophan synthetase: Many of the td mutants (including td 2 and td 71) produce a protein which is immunologically related to tryptophan synthetase of the parental wild type.[14, 6] This protein appears to be mutationally altered tryptophan synthetase and has been designated CRM (cross reacting material).

When td 2 extract is chromatographed on a DEAE cellulose ion exchange column, enzyme activity is eluted in the same fraction as the main CRM peak. In addition, Suskind and Jordan[15] have demonstrated that the enzymatic conversion of indole to InGP by td 2 extracts is inhibited by anti-CRM serum which has been absorbed by an extract of a non-CRM forming mutant.

The activity from td 71 has not been successfully chromatographed on DEAE cellulose columns, but after a 35-fold purification of the enzyme activity by conventional fractionation procedures, the ratio of CRM to enzyme activity remains constant (Table 4).

TABLE 4

RATIO OF CRM TO ENZYME ACTIVITY IN td 71 EXTRACTS

	μM/hr/mg pr	Units CRM*/mg pr	Ratio CRM/enz. act.
Crude extract	0.044	0.172	3.9
Fractionated extract	1.57	5.45	3.5

* 1 unit of CRM is defined as that amount of material which will neutralize the antibody which would inhibit 1 μM/hr of reaction (1) of wild type enzyme.

The data lend strong support to the suggestion that, in mutants td 2 and td 71, reaction (3) is catalyzed by a protein which is identical to CRM.

Characteristics of td 71 activity: Mutant td 71 has chosen for further study since it is isogenic with the wild type strain 74A. In addition, the higher activity of reaction (3) in td 71 extracts makes this mutant much more suitable for enzyme studies.

The reaction characteristics in td 71 are of interest, since all the substrates required for the conversion of InGP to tryptophan or of indole to tryptophan with wild type enzyme, are required for the conversion of InGP to indole by this mutant. This is true in spite of the fact that neither serine nor pyridoxal phosphate plays an obvious role in the enzymatic conversion in question. To investigate the possible nature of the functional alteration which has taken place, the affinity constants for serine and pyridoxal phosphate were compared to those for the wild type enzyme. The fractionated extract was used in each case. For this purpose wild type activity was assayed by reaction (1) and td 71 activity was assayed by reaction (3). The affinity constants for serine (K_A for td 71 and K_m for wild type)

were calculated from the data in Figure 1. The K_A for serine for reaction (3) in td 71 appears to be essentially the same as the K_m for serine for reaction (1) in 74A. On the other hand, the saturation curves for pyridoxal phosphate for the respective reactions are quite different (Fig. 2). The concentration of pyridoxal phosphate required for half saturation is at least 10-fold higher in the td 71 reaction. It appears that the mutational alteration which results in a loss of reactions (1) and (2) and which creates new requirements for reaction (3) also alters the pyridoxal phosphate combining site in some way. This effect on the affinity of the enzyme

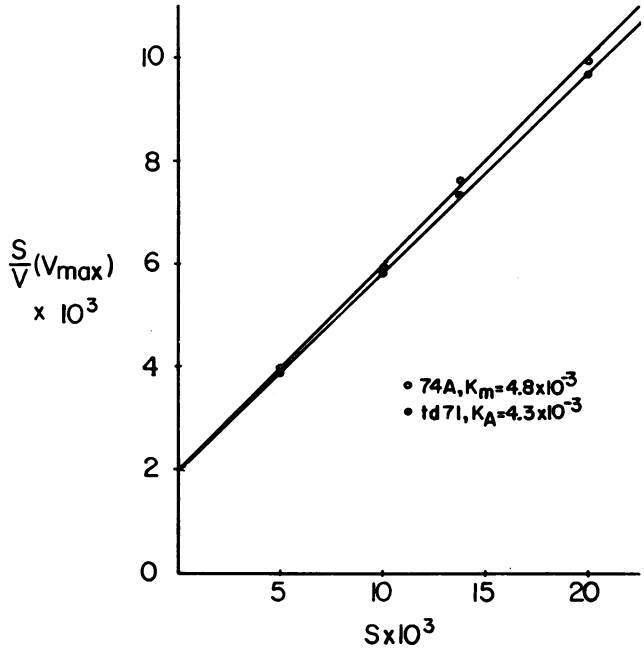

FIG. 1.—The affinity of enzyme from 74A and td 71 for serine. 74A was assayed by reaction (1) according to the procedure of Yanofsky.[9] Reaction (3) was assayed in td 71 using the complete system in Table 3. S represents the concentration of L-serine in 1.0 ml. and V the velocity of the reaction in μM per hr. Each point represents the average of determinations carried out for 3 different time intervals. The ordinate is plotted as S (V max)/V so that both curves have the same intercept and any difference in the affinity constants should be reflected in the slopes.

for pyridoxal phosphate is apparently not paralleled by an effect on the affinity of the enzyme for serine.

Discussion.—The chromatography of a *Neurospora* tryptophan synthetase on DEAE cellulose columns[16] indicates that this enzyme is a one-component system as opposed to the two-component protein system demonstrated with tryptophan synthetase from *E. coli* by Crawford and Yanofsky.[17] The single component nature of the enzyme is even more dramatically demonstrated by the relatively high state of purity obtained recently with this enzyme by Mohler and Suskind.[18] The fractionation studies presented here indicate that tryptophan synthetase in *N. crassa*, as in *E. coli*, catalyzes reactions (1), (2), and (3).

Td mutants which retain the ability to catalyze one of the tryptophan synthetase

reactions, either (1) or (3), have been isolated from *E. coli* and characterized by Yanofsky and Crawford.[19] The mutants studied here grossly resemble one of these classes. However, the requirement for pyridoxal phosphate or serine for the catalysis of reaction (3) is not exhibited by any of the *E. coli* mutants. The alterations found in td 2 and td 71 present two cases of mutation which have resulted in retention of part of the catalytic function of an enzyme, and at the same time imposed new requirements for the part of the mechanism retained.

These requirements in td 2 and td 71 pose some interesting questions regarding enzymatic mechanism since serine and pyridoxal phosphate play no obvious role in the conversion of InGP to indole. Application of current hypotheses,[20] concerning the role of pyridoxal phosphate in amino acid reactions, to the conversion of InGP or indole to tryptophan would invoke the formation of a Schiff's base between

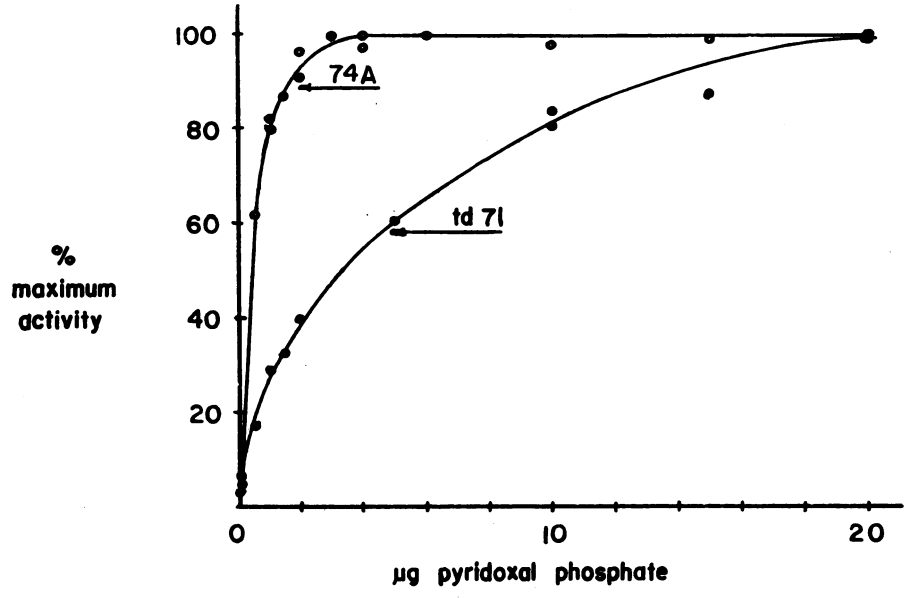

FIG. 2.—The effect of B_6P concentration on 74A and td 71 reactions. 74A was assayed by reaction (1) and td 71 reaction (3).

pyridoxal phosphate and serine on the enzyme surface which would react with the indole moiety to form tryptophan. According to this concept, the Schiff's base would not be directly involved in the conversion of InGP to indole. The absence of any requirement for pyridoxal phosphate for this conversion in wild type and the requirement for pyridoxal phosphate alone in td 2 would appear to justify this conclusion. It is possible that serine or pyridoxal phosphate are required for reaction (3) in td 2 and td 71 to maintain the active site of the altered enzyme in its "proper" physicochemical or stereospecific configuration to combine with and cleave InGP. The "induced fit" theory for enzyme action of Koshland[21] can easily be extended to explain such an activation.

It is interesting to consider what structural change could lead to the functional alterations observed. The td 71 enzyme apparently retains the ability to combine

with all of the substrates of the wild type enzyme but still fails to carry out the over-all reaction. Therefore, a simple loss or block of one of the substrate combining sites cannot be postulated. It appears that the structural alteration blocks the ability of the indole moiety to react with serine without blocking the combining sites or without blocking the catalysis of reaction (3). More information is required concerning the mechanism of the wild type enzyme conversion before any specific conclusions can be drawn. It is already apparent, however, that a detailed study of the structural and functional alterations involved in the td 2 and td 71 enzymes should provide significant information on the effect of mutation on enzyme function.

The alterations in the td 2 and td 71 enzymes are clearly different, although the gross change in the enzyme reaction is similar. The relative levels of reaction (3) present in the three strains constitute another obvious difference. The level of activity in td 2 extracts is low and is approximately that found in the wild type extracts. In addition, the CRM to enzyme activity ratio is similar in td 2 and wild type (approximately 100/1, where the units of reaction (1) are assumed to be equivalent to the units of CRM). However, in td 71 extracts the specific activity is high and approximates very closely the specific activity of reaction (2), InGP → tryptophan, found in similar preparations of wild type. The CRM to enzyme activity ratio in td 71 extracts is only about 4 to 1. Thus, not only have the requirements for this reaction been changed as a result of the mutation but there is an apparent increase in the rate of the reaction per unit of CRM activity. Alternately, a change in immunological reactivity of the protein may be involved.

Rachmeler and Yanofsky[22] have recently obtained a td mutant of *Neurospora* which is capable of catalyzing reaction (1), indole → tryptophan, but not reactions (2) or (3). A detailed analysis of the alteration involved in such a mutant should provide an interesting comparative study to the data presented here.

Summary.—Only two of the many td mutants isolated from *Neurospora crassa* accumulate indole in their growth filtrates. While wild type tryptophan synthetase catalyzes reactions (1), (2), and (3), extracts from these two mutants, td 2 and td 71, catalyze reaction (3), but not reactions (1) or (2). Experimental evidence to date indicates that reaction (3), in extracts from td 2 and td 71, is catalyzed by a protein (CRM) which is immunologically related to wild type tryptophan synthetase. It is therefore suggested that the reaction is catalyzed by a mutationally altered tryptophan synthetase in these mutants.

These studies indicate that, while the ability to carry out reaction (3) is retained, something more subtle than loss of combining sites for serine and for pyridoxal phosphate is involved in the loss of reactions (1) and (2). While reaction (3) in wild type does not require pyridoxal phosphate and serine for the conversion of InGP to indole, reaction (3) in td 2 requires pyridoxal phosphate and reaction (3) in td 71 requires both pyridoxal phosphate and serine. Thus, in addition to loss of ability to catalyze reactions (1) and (2), the altered enzymes have acquired some new requirements for the reaction retained.

In more detailed studies with td 71 extracts, it was found that the association constant for serine is very similar to the K_m for serine in the wild type reaction. On the other hand, the concentration curves for pyridoxal phosphate are significantly different for the td 71 and the wild type reactions.

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*MEIOTIC DRIVE IN NATURAL POPULATIONS OF DROSOPHILA MELANOGASTER. II. GENETIC VARIATION AT THE SEGREGATION-DISTORTER LOCUS**

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In a previous report, Sandler, Hiraizumi, and Sandler[1] have presented an account of the elementary observations concerning the phenomenon of segregation-distortion. Briefly, these observations are as follows: *Segregation-distorter* (symbol *SD*) is a locus, located in or near the centromeric heterochromatin of chromosome II, which was discovered in a natural population of *D. melanogaster*. When *SD* is made heterozygous with a structurally normal, non-*SD*-bearing, second chromosome in a male, the *SD*-bearing chromosome is recovered very much more often than its (SD^+-bearing) homolog. If the ratio of *SD*-carrying progeny to the total progeny in a backcross, $SD/+$ ♂♂ × $+/+$ ♀♀, is called k, then, in many experiments, the average k value is 0.95 or greater. When, however, *SD* is made heterozygous in males carrying, as a homolog for *SD*, a structurally rearranged SD^+-bearing chromosome (as, for example *In* (*2LR*) *Cy*); the phenomenon is suppressed, and the k value is approximately 0.50. Data of this kind have been interpreted to mean that an *SD* locus must pair with an SD^+ locus in order for segregation-distortion to occur. In SD/SD^+ females, it should be mentioned, there is no segregation-distortion under any circumstances.