FACTORS AFFECTING INCREASED PRODUCTION OF TRYPTOPHAN SYNTHETASE BY A TD MUTANT OF *NEUROSPORA CRASSA*

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ABSTRACT

MATCHETT, WILLIAM H. (University of California, San Diego, La Jolla, Calif.) AND JOHN A. DEMOSS. Factors affecting increased production of tryptophan synthetase by a td mutant of *Neurospora crassa*. J. Bacteriol. **83**:1294–1300. 1962.—Mutant td 201 of *Neurospora crassa* produces a tryptophan synthetase in significantly larger amount than the parental wild-type strain. Evidence obtained in the present investigation indicates that tryptophan synthetase formation in this mutant is subject to end product regulation by a pool of internal tryptophan. An accelerated rate of formation of enzyme was observed when the level of internal tryptophan fell below the value of 1 μmole per g dry weight of mycelium. It is concluded, therefore, that the high level of enzyme found in this strain results from release of repression. In experiments with the parental wild-type strain, no such release of repression was observed. The significance of these results for the problem concerning the nature of the mutation giving rise to mutant td 201 is discussed.

Evidence indicating a marked effect of growth temperature upon metabolism of externally supplied tryptophan is presented.

The tryptophan synthetase from *Neurospora crassa* has been the subject of recent extensive biochemical and genetic studies (Yanofsky, 1960; Bonner, Suyama, and DeMoss, 1960).

Most strains of *Neurospora*, both wild types and td mutants (strains lacking tryptophan synthetase or producing an altered form of the enzyme), produce relatively small amounts of tryptophan synthetase. Lester (1961*a*,*b*) has demonstrated that in certain tryptophan auxotrophs the specific activity of tryptophan synthetase is two- to threefold higher in conidia germinated in the absence of exogenous tryptophan than in conidia germinated in the presence of exogenous tryptophan. Since similar fluctuations could not be effected in a wild-type strain, he concluded that in the wild-type strain the tryptophan synthetase-forming system is fully repressed by endogenously formed tryptophan, though in tryptophan auxotrophs the system becomes derepressed when tryptophan is exhausted. Although these conclusions may be valid for the relatively homogenous germinating-conidia system, we have not been able to obtain significantly higher levels of tryptophan synthetase activities in fully grown mycelia of tryptophan auxotrophs grown under standard laboratory conditions (i.e., at 30 C for 72 hr), even though the exogenous tryptophan is exhausted. However, one strain (mutant td 201), described in some detail by Rachmeler and Yanofsky (1961), was shown to produce an altered tryptophan synthetase in significantly larger amounts than wild-type strains produce normal enzyme. Since td 201 appears to be unique in this respect, the present study was undertaken to determine what factors are involved in the production of a high level of tryptophan synthetase by this strain.

MATERIALS AND METHODS

Wild-type strain 5256 and mutant td 201 of *N. crassa* were used in this investigation. Of the three reactions (DeMoss and Bonner, 1959) catalyzed by the wild-type enzyme, the mutant enzyme has the ability to catalyze only one, the conversion of indole to tryptophan (Rachmeler and Yanofsky, 1961). As indicated below, disappearance of indole in this reaction was used as the criterion of enzymatic activity.

Stock cultures of these strains were maintained on agar slants prepared from the minimal medium of Vogel (*unpublished data*). L-Tryptophan (150 μg/ml) was added to support growth of the mutant strain. Inocula for liquid cultures were prepared by suspending conidia of slant cultures in sterile distilled water.

Experimental mycelia were grown in liquid

minimal media. To support growth of the mutant strain, minimal medium was supplemented either with indole (20 μg/ml) or with L-tryptophan (150 μg/ml). Cultures (2 liters) were grown in 4-liter glass bottles. Cultures (15 liters) were grown in 20-liter glass carboys. The cultures were grown with forced aeration and incubated in water baths maintained at 20 or 30 C. Mycelia were harvested by filtration of the culture through cheesecloth and were washed with distilled water. The washed mycelia were stored at −15 C.

Crude extracts were prepared by the method of Yanofsky (1955).

Enzyme activity was measured by observing the disappearance of indole (Yanofsky, 1955). Inorganic pyrophosphatase activity was determined by measuring inorganic phosphate liberated from sodium pyrophosphate by the method of Fiske and SubbaRow (1925). Tryptophan was measured with the tryptophanase reaction (DeMoss, *in press*). Protein was precipitated from crude extracts with 5% trichloroacetic acid, redissolved in 0.1 M NaOH, and measured with the Folin reagent (Lowry et al., 1951).

Internal tryptophan was determined in the following way. Lyophilized powdered mycelium (1 g) was extracted with 15.0 ml of 0.05 M KPO_4 buffer (pH 7.8) for a period of 30 min at 0 C. The resulting paste was centrifuged at 40,000 × *g* for 30 min. A 2-ml sample of the supernatant fluid was heated at 100 C in a boiling-water bath for 2 min to inactivate tryptophan synthetase. This sample was then extracted with 4.0 ml of toluene to remove any trace of indole. A 1-ml sample of the aqueous layer resulting from this extraction was removed and its tryptophan content determined by treatment with tryptophanase and measurement of the indole thus formed. The assumption that the values so obtained represent internal tryptophan and not tryptophan from the medium contaminating the surface of the mycelium is substantiated by the following considerations. (i) In tryptophan-supplemented cultures grown at 30 C, the internal concentration was found to vary independently from the external concentration during the period of rapid uptake of the amino acid from the medium. (ii) The internal concentration in these mycelia remained at a level higher than 4 μmoles per g dry weight of mycelium for at least 60 hr after tryptophan had been exhausted from the external medium.

RESULTS

An experiment designed to test the effects of cultural temperature and growth supplement upon specific activity of tryptophan synthetase activity of strain td 201 gave the data presented in Table 1. Of the four cultures studied, the one supplemented with tryptophan and incubated at 30 C gave mycelium with the lowest enzyme specific activity. The cultures supplemented with indole and incubated at either temperature gave values which were threefold higher, and the culture supplemented with tryptophan and incubated at 20 C gave a value fivefold higher than the lowest value obtained. These data suggest that externally supplied tryptophan represses the formation of tryptophan synthetase at 30 C but not at 20 C.

Table 1 shows only the end results obtained from cultures incubated for a period of 72 hr. The curves of Fig. 1 and 2 show the results obtained from experiments in which enzyme specific activity was followed throughout the culture period. Samples of mycelium and filtrate were

TABLE 1. *Effects of cultural temperature and growth supplement upon specific activity of tryptophan synthetase present in crude extracts of mutant td 201*[a]

Growth supplement[b]	Growth temperature	Specific enzyme activity[c]
	C	
Tryptophan	20	1.5
Tryptophan	30	0.3
Indole	20	0.9
Indole	30	1.0

[a] Cultures were incubated in water baths maintained at either 20 or 30 C for a period of 72 hr.

[b] Initial concentration of tryptophan = 0.75 μmole/ml; initial concentration of indole = 0.18 μmole/ml.

[c] Enzyme activity was determined by measuring the disappearance of indole (Yanofsky, 1955). The complete mixture contained (in μmoles): KPO_4 buffer (pH 7.8), 10; L-serine, 40; indole, 0.5; glutathione, 0.25; and pyridoxal phosphate 0.05; in a final volume of 1.0 ml. Specific activity equals units of enzyme per mg protein; 1 unit of enzyme activity equals that amount of enzyme which will catalyze the disappearance of 1.0 μmole of indole in 1 hr at 37 C.

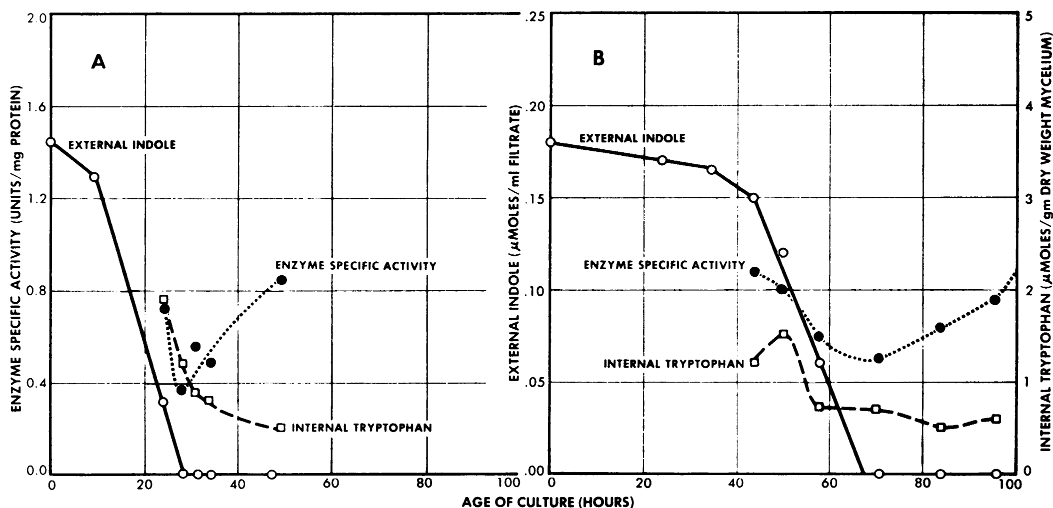

FIG. 1. *Effect of temperature on tryptophan synthetase specific activity and internal concentration of tryptophan in mycelium cultured in indole-supplemented media (A = 30 C; B = 20 C). Indole remaining in the filtrate was measured by the method of Yanofsky (1955). Internal tryptophan was extracted from lyophilized powdered mycelia and measured with the tryptophanase reaction (DeMoss, in press). See Table 1 and Materials and Methods for measurement of enzyme specific activity.*

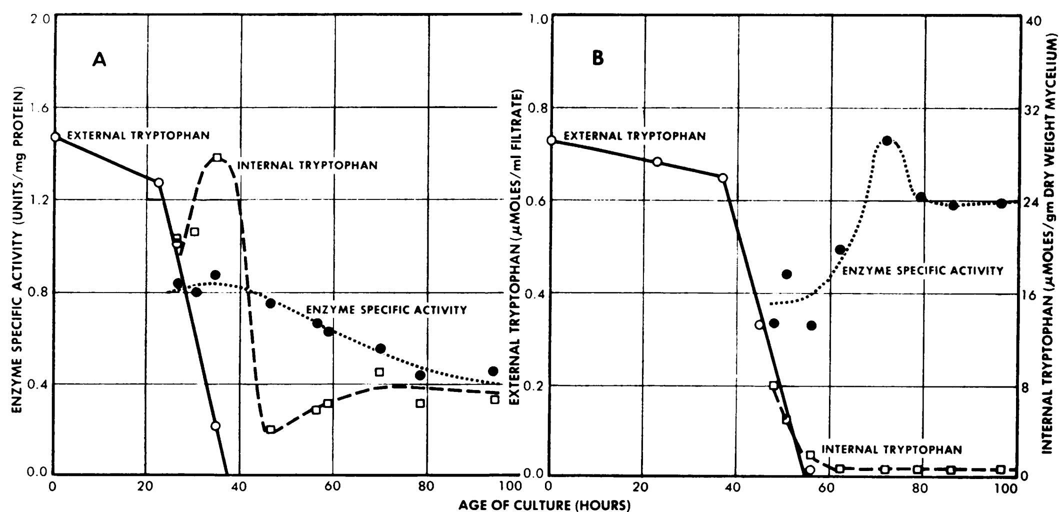

FIG. 2. *Effect of temperature on tryptophan synthetase specific activity and internal concentration of tryptophan in mycelium cultured in tryptophan-supplemented media (A = 30 C; B = 20 C). Tryptophan remaining in the filtrate and buffer-extractable tryptophan were measured with the tryptophanase reaction (DeMoss, in press). See Table 1 and Materials and Methods for measurement of enzyme specific activity.*

removed periodically from 15-liter cultures incubated in water baths maintained at either 20 or 30 C. Supplement remaining in the filtrate, tryptophan extractable from the lyophilized mycelium, and the specific activity of tryptophan synthetase were determined for each pair of samples. Figure 1 shows the results obtained from the pair of cultures supplemented with indole. At either temperature studied, the time of indole exhaustion from the filtrate corresponded closely to the time when buffer-extractable tryptophan fell below a value of 1 μmole per g dry weight. This

time also corresponded closely to the time when enzyme specific activity reached a minimal value and began to increase. These data may be interpreted in the following way. Indole supplied from the medium is converted to tryptophan by an initially high level of tryptophan synthetase. This conversion occurs at a rate greater than that at which tryptophan is utilized for growth, resulting in the formation of a pool of internal tryptophan. The presence of this pool results in end product repression of the formation of tryptophan synthetase. When the external supply of indole limits the rate of formation of tryptophan, the internal concentration of this amino acid is reduced to a level below 1 μmole per g dry weight of mycelium. When this occurs, end product repression is released, resulting in the observed increase in enzyme specific activity.

Figure 2 depicts the results obtained from cultures supplemented with tryptophan and incubated at either 20 or 30 C. Data obtained from the culture incubated at 30 C reveal that the internal concentration of tryptophan rose to the relatively high level of 28 μmoles of tryptophan per g dry weight of mycelium during the period of rapid uptake of this supplement from the medium. Immediately after the disappearance of tryptophan from the medium, the internal concentration fell to a low value of 4 μmoles per g dry weight mycelium and slowly rose to a steady value of about 8 μmoles per g. Samples of filtrate taken during this period supported growth of the wild-type strain. This fact demonstrates that the medium was not exhausted with respect to nutrients other than tryptophan. The continued presence of this pool of internal tryptophan apparently results in a diminution in the relative rate of formation of tryptophan synthetase, as evidenced by the slow decline in specific activity of the enzyme. Data obtained from the culture incubated at 20 C present a different picture. During the period of rapid supplement utilization, the concentration of internal tryptophan fell from a high value of 8 μmoles per g dry weight of mycelium to a steady low value significantly less than 1 μmole per g dry weight. Enzyme specific activity rose abruptly, starting at the time when the concentration of internal tryptophan fell below 1 μmole per g of mycelium and reached a steady high value about threefold higher than the low value obtained from the culture incubated at 30 C.

Apparently tryptophan regulates the formation of tryptophan synthetase in the growing mycelium of td 201 by exerting end product repression at internal concentrations greater than 1 μmole per g dry weight of mycelium. Cultural temperature has no effect upon enzyme formation in cultures supplemented with indole. It would appear, therefore, that cultural temperature has an indirect effect upon enzyme formation in cultures supplemented with tryptophan. The direct effect of temperature in tryptophan-supplemented cultures appears to be the regulation of the concentration of internal tryptophan. At 30 C the internal concentration of this supplement is high with respect to its concentration at 20 C.

The nature of the mycelial mass makes it impossible to estimate, from samples, the total growth of a liquid culture of *Neurospora*. For this reason total enzyme could not be calculated from the data of Fig. 1 and 2. The following experiment illustrates that changes in specific activity result from changes in total activity (Table 2). Since the inoculum used in this experiment consisted of mycelium rather than conidia it was possible to determine precisely the total amount of protein and enzyme introduced into each culture at zero time of the culture period. After the incubation period, the entire culture was harvested. Total enzyme and total protein were then determined. With this information, it was possible to calculate increases which occurred during the period of incubation.

Data obtained from the pair of cultures supplemented with tryptophan show that the increase in specific activity observed in the culture incubated at 20 C is the result of increased synthesis of tryptophan synthetase. At 20 C, 1,000 units of enzyme and 656 mg of protein were synthetized, whereas at 30 C only 570 units of enzyme were obtained during a synthesis of 1,250 mg of protein. This difference in the relative rate of formation of enzyme resulted in the threefold increase in specific activity observed in the 20-C culture. In addition, these data show that although equal quantities of tryptophan were removed from the medium at each temperature studied, nearly twice as much protein was synthesized at 30 as at 20 C. This fact, which has been confirmed in repeated growth experiments, may be interpreted as evidence for a difference in the metabolism of externally supplied tryptophan at 20 and 30 C. It would appear that, at 20 C, less tryptophan is available for protein synthesis and growth than at 30 C. It is possible

TABLE 2. *Effects of temperature and supplement upon formation of tryptophan synthetase*[a]

Growth supplement	Growth temperature	Total protein	Total enzyme	Specific activity[b]	Protein synthesized	Enzyme synthesized	Specific activity enzyme synthesized	Supplement concn (μmole/ml)		Tryptophan internal concn (μmole/g dry wt)[c]
								Initial	Final	
	C	*mg*	*units*	*units/mg*	*mg*	*units*	*units/mg*			
Tryptophan	20	745	1,050	1.41	656	1,000	1.52	0.73	0.0	1.1
	30	1,340	616	0.46	1,250	570	0.46	0.73	0.0	10.2
Indole	20	616	647	1.05	527	601	1.14	0.18	0.0	0.6
	30	756	1,250	1.65	667	1,200	1.80	0.18	0.0	0.3
None	20	743	1,030	1.38	108	700	6.48	—	—	0.6
	30	800	1,100	1.38	165	770	4.66	—	—	0.6

[a] Cultures (2 liters) were inoculated with samples of mycelium harvested from a 15-liter culture grown 48 hr at 30 C on medium supplemented with tryptophan. Each of the tryptophan- and indole-supplemented cultures was inoculated with a sample of mycelium containing 89 mg protein, 46 units of enzyme, and 3.1 μmoles extractable tryptophan per g dry weight of mycelium. Each of the minimal medium cultures received an inoculum containing 635 mg protein, 330 units of enzyme, and 3.1 μmoles tryptophan per g dry weight of mycelium.

[b] See Table 1 and Materials and Methods.

[c] See Fig. 1 and Materials and Methods.

that at 20 C an additional tryptophan-utilizing pathway is induced or activated which competes for available tryptophan with protein synthesis. It is also possible that the operation of this pathway results in the lower concentration of internal tryptophan noted in Fig. 2. In any case, it seems clear that the direct effect of temperature in these cultures is exerted upon the internal tryptophan pool, which in turn regulates the formation of tryptophan synthetase.

That the temperature effect is confined to the metabolism of externally supplied tryptophan is illustrated by the data obtained from the cultures supplemented with indole. In each of these cultures, tryptophan arose endogenously from externally supplied indole, and no significant difference in the amount of protein synthesized was observed. That the culture incubated at 30 C synthesized roughly twice the amount of enzyme synthesized at 20 C may be explained by the fact that indole disappeared from the former after 9 hr of incubation, whereas indole did not disappear from the latter until 18 hr of incubation had elapsed. The curves of Fig. 1 indicate that repression is released soon after the disappearance of indole from the medium. Consequently, the 30 C culture experienced 13.5 hr under conditions of derepression whereas the 20-C culture experienced only 4.5 hr under these conditions.

The data obtained from the unsupplemented cultures illustrated that accelerated formation of tryptophan synthetase, resulting from release of repression, can occur in mycelia under conditions which do not permit significant net increases in protein.

The objection could be raised that the observed decrease in enzyme activity is due not to end-product repression of the synthesis of enzyme but to the presence of an inhibitor of the enzyme activity in extracts of "repressed" mycelium. This possibility was eliminated by the results of an experiment in which extracts of repressed and derepressed mycelia were measured separately and mixed together in various combinations. Activities were found to be additive in all cases. No stimulation or inhibition of activity was observed when boiled extract of either kind was added to unboiled extract of either kind.

As a second control for this experiment, activity of the enzyme inorganic pyrophosphatase was measured in extracts of the inoculum and each of the experimentally cultured mycelia. These measurements revealed that in the tryptophan- and indole-supplemented cultures increases in protein were paralleled by increases in inorganic pyrophosphatase activity. The minimal medium cultures lost activity during the incubation period. These results indicate that the observed increases in tryptophan synthetase activity were not due to some nonspecific effect on enzymes in general.

It was mentioned that Rachmeler and Yanof-

TABLE 3. *Effects of temperature and externally supplied tryptophan upon specific activity of tryptophan synthetase present in crude extracts of wild-type strain 5256*[a]

Supplement	Culture temperature	Specific enzyme activity[c]	Tryptophan internal concentration (μmoles/g mycelium)[d]
	C	*units/ml*	
Tryptophan...	20	0.35	1.4
Tryptophan...	30	0.24	16.6
None..........	20	0.18	0.2
None..........	30	0.18	0.6

[a] Cultures were incubated in water baths maintained at either 20 or 30 C for a period of 80 hr.

[b] Initial concentration of tryptophan = 0.75 μmole/ml.

[c] See Table 1 and Materials and Methods.

[d] See Fig. 1 and Materials and Methods.

sky (1961) observed that mutant td 201 produces significantly higher levels of altered tryptophan synthetase than those observed in typical wild-type strains of *Neurospora*. Our data indicate that this high level of enzyme results at least in part from release of repression and that maximal derepression occurs in mycelium grown in tryptophan-supplemented medium at 20 C. The possibility was considered, therefore, that these conditions of growth might produce a similar response in wild-type mycelium. To test this possibility an experiment, similar in design to the one described in Fig. 2, was performed with wild-type mycelium. The data obtained from the 80-hr samples are presented in Table 3. The specific enzyme activity values for these samples are typical, and significant departures from these values were not observed during this experiment. Tryptophan uptake by this strain was remarkably similar to that observed in td 201 (Fig. 2). These data confirm the observation in the mutant strain that there is a large difference in the internal tryptophan concentrations of mycelia cultured at 20 and 30 C in the presence of tryptophan. In spite of this difference, a relatively small variation in enzyme specific activity was observed. The internal concentration of tryptophan observed in the culture supplemented with tryptophan and incubated at 30 C was 30-fold higher than the concentrations observed in either of the minimal medium cultures, but no significant difference in specific activity of the enzyme was observed in any of these cultures. These observations do not rule out the possibility that the level of enzyme in the wild-type strain is subject to end-product regulation by tryptophan. They do show, however, that variations in the concentration of internal tryptophan which have a marked effect upon enzyme level in td 201 are without effect in the wild-type strain.

DISCUSSION

Lester (1961*a*,*b*) has recently presented evidence indicating that externally supplied tryptophan represses the formation of tryptophan synthetase in germinated conidia of tryptophan auxotrophs of *Neurospora*. The data obtained in the present investigation confirm and extend his conclusions. They demonstrate that formation of the enzyme by growing mycelium of td 201 is repressed when the concentration of internal tryptophan exceeds 1 μmole per g dry weight of mycelium. When the concentration of internal tryptophan falls below this level, repression is released and formation of enzyme proceeds at an accelerated rate. The high level of enzyme observed in this strain is, apparently, the result of release of repression.

Formation of enzyme by wild-type strain 5256, on the other hand, is not similarly influenced. The present data demonstrate clearly that concentrations of internal tryptophan which have a marked effect on enzyme formation in the mutant are without effect on enzyme formation in the wild-type strain. An experiment with a revertant of td 201 gave essentially the same results as those reported in Table 3.

It would appear, from a consideration of these findings, that the lesion giving rise to mutant td 201 is phenotypically more complex than was originally supposed. In addition to causing a change in the catalytic properties of the enzyme, this mutation appears to have resulted in an alteration of the regulatory mechanism controlling the rate of formation of the enzyme.

A precise interpretation of the effect of temperature upon metabolism of externally supplied tryptophan observed in this work must await more information. Tentatively, it may be assumed that at 20 C tryptophan serves as a substrate not only for protein synthesis but also for additional reactions leading to its degradation or conversion to products not available for protein synthesis. It would appear that at 30 C these

reactions are either absent or quantitatively negligible in terms of reduction of internal tryptophan concentration.

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