SOME ASPECTS OF TRYPTOPHAN SYNTHETASE FORMATION IN *NEUROSPORA CRASSA*[1]

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The regulation of metabolic sequences by their end products through inhibition of an early enzymatic reaction or of enzyme formation (repression) have become well recognized phenomena, which are being increasingly explored. The bulk of the present information on feedback inhibition and repression has been derived from work with bacterial systems. Although it has been variously pointed out (Pollock, 1959; Pardee, 1959)[2] that such regulatory mechanisms might play an important role in differentiative processes, relatively little work has been done with organisms more morphologically differentiated than bacteria. For instance, a recent compilation of examples of feedback inhibition (Umbarger, 1961) contains 2 nonbacterial systems out of 13 listed; a listing of 27 examples of repression (Vogel, 1961) contains only one system in a mold and two occurring in cells of higher organisms. Perhaps a major deterrent to working with more complex organisms is that their cultural characteristics do not appear to offer an opportunity for the elegant kinetic work which has been done with bacteria. However, an assessment of the roles of feedback inhibition and repression in the processes of differentiation, as well as their ubiquity, demands that these regulatory mechanisms be studied in organisms other than bacteria. From this standpoint studies have been instituted on the regulatory aspects of tryptophan formation by *Neurospora crassa*, and this paper presents the initial results of these studies.

N. crassa has been the subject of extensive biochemical and genetic investigations which should provide a background for studies on regulatory mechanisms. The tryptophan system has been selected for examination since the pathway of tryptophan biosynthesis is well defined, and many mutants are available which permit the isolation of various segments of tryptophan biosynthesis (Bonner and Yanofsky, 1951). Also, the tryptophan system has been well delineated both biochemically and genetically in *Escherichia coli* (Yanofsky, 1960), and the influence of tryptophan on this system with respect to feedback inhibition and repression has received some attention (Lester and Yanofsky, 1961); this should provide for a comparison of the regulation of tryptophan biosynthesis in organisms of diverse complexity. This paper deals with an examination of the last step in tryptophan biosynthesis, namely, the formation of tryptophan synthetase activity.

[1] This work was supported by a grant from the Commonwealth Fund.

[2] See also papers by M. Cohen, L. Gorini and W. K. Maas, H. J. Vogel, and B. Magasanik, in *The chemical basis of development*, pp. 458–490 (1958), edited by W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore.

MATERIALS AND METHODS

Organisms. Two strains of *N. crassa* were used in these studies: strain 74A, which is tryptophan independent, was obtained from D. M. Bonner, and strain 10575A, which requires either indole or tryptophan for growth and accumulates anthranilic acid (Tatum, Bonner, and Beadle, 1944), was obtained from S. R. Suskind. Stock cultures of these strains were maintained on a medium devised by H. S. Vogel (Lester and Hechter, 1961).

Production of conidia. The methods and medium for obtaining large numbers of conidia have been described elsewhere (Wainwright, 1959), and will be summarized only briefly. Cultures were grown on a chemically defined agar medium (200 ml per 1-liter Erlenmeyer flask, or 400 ml per 2.8-liter Fernbach flask), supplemented with 100 μg L-tryptophan per ml in the case of strain 10575A. After 4 to 5 days of incubation at 30 C, the cultures were held at room temperature under continuous illumination for another 5 to 6 days, and then stored at about 4 C until used.

Preparation of germinated conidia. Conidia were scraped from the cultures into distilled

water and shaken vigorously. The suspension was then filtered several times through increasing thicknesses of cheese cloth (1 to 16 layers), and then through a pad of glass wool, to remove associated mycelia. The conidia were washed once with a large volume of distilled water. For germination, half-strength Fries medium (Beadle and Tatum, 1945) containing 0.5% sucrose and other desired supplements was used. The medium was inoculated with 2.5 to 2.8 $\times$ 10^7 conidia per ml and incubated at room temperature (about 22 C) for 16 to 18 hr on a rotary shaker. The germinated conidia were harvested by centrifugation, and washed 3 to 4 times with a saline-phosphate buffer (0.05 M NaCl in 0.02 M NaH_2PO_4, adjusted to pH 5.8 with NaOH). Under these conditions essentially all of the conidia germinated, but the growth of the hyphae was so limited that extensive clumping did not occur. A fairly heavy suspension (10 to 15 mg dry weight per ml) could be handled with a pipette with a large bore (2 to 2.5 mm diameter) at its tip.

Chemicals. The compounds used in these studies were obtained from commercial sources.

Tryptophan synthetase. The assay of tryptophan synthetase activity in whole cells was based on the disappearance of indole from suspensions of germinated conidia in saline-phosphate buffer (described above) containing 0.4 to 0.8 μmole indole, 5.0 mg DL-serine, and 5 μg pyridoxal phosphate per ml. Usually, the serine was added 20 to 30 min before the other components. The assay mixture was incubated in a water bath at 30 C with agitation; ordinarily, a cell concentration of 2 to 4 mg dry weight per ml was used. At intervals samples were taken and 1.0-ml portions were added to 0.5 ml of saline-phosphate buffer plus two drops of 0.5 M K_2CO_3. Samples were extracted with 1.0 ml of toluene for each 0.1 μmole indole initially present, and the toluene extracts were assayed for indole (Yanofsky, 1955). Since the disappearance of indole was practically directly proportional to incubation time or cell concentration, the unit of tryptophan synthetase was designated as the disappearance of 1.0 μmole of indole per 3 hr at 30 C, and the specific activities as units per mg dry weight of cells. It may be noted here that essentially the same results were obtained whether whole suspensions, heated or unheated prior to extraction, or filtrates were used.

The determination of tryptophan synthetase activity in cell extracts was also based on indole disappearance (Yanofsky, 1955). Suspensions of germinated conidia were filtered and rinsed with cold buffer (0.02 M Na_2HPO_4, adjusted to pH 7.8 with HCl) and stored at -20 C overnight. The frozen cells were ground in a chilled Ten Broeck mill with a small amount of buffer containing 20 μg pyridoxal phosphate and 1 μmole glutathione per ml. The homogenate was centrifuged at 6,000 rev/min for 30 min and the supernatant was assayed for tryptophan synthetase activity; the assay mixture, in addition to the cell extract, contained 0.4 μmole indole, 4.0 mg L-serine, 20 μg pyridoxal phosphate, and 1.0 μmole glutathione per ml of 0.1 M Na_2HPO_4, adjusted to pH 7.8 with HCl. Incubation was carried out at 30 C, and 1.0-ml samples were withdrawn at intervals up to 60 min and extracted with 4.0 ml of toluene. The toluene extracts were assayed for indole. Indole uptake was proportional to time and the amount of extract used; the unit of tryptophan synthetase for cell extracts was designated as the disappearance of 1.0 mμmole of indole per hr, and the specific activity as units per mg extract protein.

Determination of protein and tryptophan. Protein determinations (Lowry et al., 1951) were made on cell extracts, using Armour bovine-plasma, fraction IV, as a reference standard. Tryptophan determinations (Nason, Kaplan, and Colowick, 1951) were made in conjunction with some of the estimations of tryptophan synthetase by indole uptake, using the aqueous phase after two extractions with toluene (the first extract was used for indole assay). In some cases where tryptophan or its analogues were added to the germination medium, the culture filtrates were assayed for tryptophan; the culture filtrates were first made acid (pH 2) and extracted with ethylacetate to remove acidic indolyl compounds, such as indole-3-acetic acid (Tatum and Bonner, 1944).

RESULTS

Effect of serine and pyridoxal phosphate on indole uptake by germinated conidia. The conversion of indole to tryptophan by tryptophan synthetase involves a condensation of indole and serine, with pyridoxal phosphate acting as coenzyme (Yanofsky, 1952). Consequently, if indole uptake by germinated conidia is to be representative of tryptophan synthetase activity, a requirement for serine should be observed. A

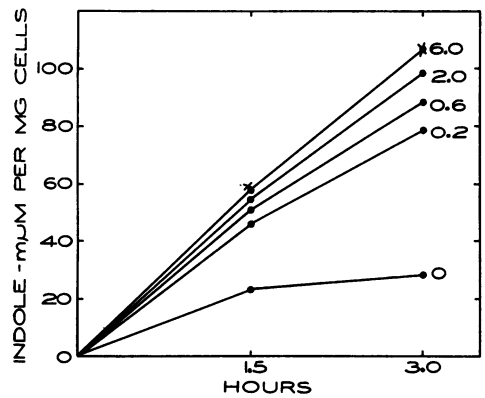

Fig. 1. Effect of serine and pyridoxal phosphate on indole uptake by germinated conidia. The numbers associated with the curves represent the initial concentration of DL-serine in milligrams per ml. The crosses (X) indicate the uptake of indole with an initial concentration of 6.0 mg DL-serine per ml, but no added pyridoxal phosphate. Otherwise, 10 μg pyridoxal phosphate per ml were present.

requirement for pyridoxal phosphate was not expected since in early studies (Tatum and Bonner, 1943, 1944) on tryptophan synthesis by Neurospora, the condensation of indole and serine by mycelia did not exhibit a requirement for pyridoxal phosphate. Fig. 1 shows that in the absence of added serine the uptake of indole is slow, initially, and then ceases. With increasing concentration of serine the rate of indole uptake increases and is almost linear with time at concentrations above 2 mg per ml. In this experiment, unlike the others to follow, the cells were not preincubated with serine before adding indole; preincubation with serine gives a more linear rate of indole uptake. The uptake of indole observed in the absence of added serine could be due to endogenous serine, either in an amino acid pool or arising from protein degradation.

The serine requirement for indole uptake by germinated conidia appears to be lower than that observed with purified tryptophan synthetase preparation (Yanofsky, 1952). This difference can be attributed to the ability of these cells to concentrate serine (*unpublished data*). However, a continuing uptake of indole by whole cells is dependent on the concomitant presence of serine, and it may be assumed that indole uptake represents tryptophan synthetase activity. (The validity of this assumption will be supported further by the correspondence of indole uptake by whole cells and extracts in experiments below.) Fig. 1 also indicates that pyridoxal phosphate is not essential for indole uptake by whole cells, and, presumably, there is a sufficient endogenous supply. Nevertheless, pyridoxal phosphate (5 μg per ml) was added in all the experiments which follow.

Effect of cell concentration. Table 1 shows that the uptake of indole is directly proportional to the concentration of germinated conidia, as well as being linear with time. Estimates of indole uptake were made by two procedures in this experiment: (i) extraction of the cell-free filtrate of the reaction mixture, and (ii) extraction of the whole reaction mixture. The values obtained were the same in both cases, and, consequently, indicate that indole is not accumulated by these cells to an appreciable extent. Thus, its disappearance from the medium represents the metabolism of indole, presumably via tryptophan synthetase action. These two experiments permitted the designation of the unit of tryptophan synthetase activity as the disappearance of 1.0 mμmole of indole per 3 hr, and the comparison between activities of reaction mixtures with different cell concentrations on the basis of units per mg dry weight.

Effect of tryptophan during germination on formation of tryptophan synthetase. To determine whether tryptophan can repress the formation of tryptophan synthetase, conidia were germinated in two media, one containing no added tryptophan, the other supplemented with 1.0 μmole of L-tryptophan per ml. The dry weight of the inoculum amounted to 0.8 mg per ml and the dry weight yield of germinated conidia was 2.9 to 3.0 mg per ml in both cases, or about a 3.5-fold increase in cell mass. Cells from such cultures exhibit no appreciable difference in tryptophan synthetase activities; the specific activities were 85 and 80 for cells grown in the absence and presence of L-tryptophan, respectively.

Although these results suggest that tryptophan does not repress tryptophan synthetase formation, the possibility was considered that the expression of a repressive action depended on a longer exposure to tryptophan or a greater increment of growth, or both. This possibility was examined by varying the inoculum size of the germination cultures; thus, with fewer conidia there would be greater growth and longer exposure to tryptophan on the part of an

TABLE 1

Effect of cell concentration on indole uptake

Cell Concn	Incubation Time	Indole Uptake			
		Suspension		Filtrate	
mg/ml	*hr*	*mμmoles/ml*	*mμmoles/mg*	*mμmoles/ml*	*mμmoles/mg*
1.9	1.5	79	42	79	42
1.9	3.0	165	87	161	84
3.7	1.5	146	40	158	43
3.8	3.0	318	86	329	89
5.6	1.5	236	42	217	39
5.8	3.0	491	88	476	85

TABLE 2

Effect of inoculum size on tryptophan synthetase formation in absence and presence of tryptophan

Inoculum		L-Tryptophan	Germinated Conidia	Tryptophan Remaining*	Tryptophan Synthetase
Conidia per ml					
no. × 10⁷	*mg*	*μmole/ml*	*mg/ml*	*μmole/ml*	*specific activity*
2.8	0.84		3.2	Nil	89
1.4	0.47		3.1	Nil	95
0.7	0.21		2.6	Nil	108
2.8	0.84	1.0	3.1	0.09	96
1.4	0.42	1.0	2.5	0.06	105
0.7	0.21	1.0	1.8	0.06	114

* Determined in culture filtrates after germination of conidia.

individual conidium. The results of such an experiment are given in Table 2. Here it can be seen that, by varying the inoculum size, 3.7- to 12.4-fold increases in cell mass are obtained during germination. The presence of tryptophan appears to cause an inhibition of growth when low levels of inocula are used, but the nature of this inhibitory effect is not known yet. There is a tendency for a higher tryptophan synthetase activity in cells derived from a lower inoculum; it might be mentioned here that conidia also exhibit tryptophan synthetase activity of about 60 to 70 units per mg dry weight. However, the presence of tryptophan in the germination medium has no effect on the formation of tryptophan synthetase activity. This indicates that a repressive action of tryptophan does not depend on the duration of growth in the presence of tryptophan.

Effect of tryptophan analogues and precursors during germination on formation of tryptophan synthetase. When conidia are germinated in the presence of tryptophan, under the conditions described, the medium is found to contain only 5 to 10% of the tryptophan initially present (Table 2). However, as will be shown, maximal yields of cell mass of a tryptophan dependent strain are obtained with about 0.2 μmole per ml added. This suggested that degradation via kynurenin or other metabolic alterations, such as conversion to indoleacetic acid, might modify a possible repressive effect of tryptophan. In the hope of circumventing such a situation, some analogues and precursors of tryptophan, which might be less readily metabolized or serve as a continuous source of endogenous tryptophan, were examined for an effect on tryptophan synthetase formation. The results, given in Table 3, show that only 6-methyltryptophan inhibited growth, and none of the compounds reduced the tryptophan synthetase activity. On the contrary, with the exception of tryptophan, all produced increased tryptophan synthetase activities. The basis for these stimulatory effects is not known as yet. However, the possibility of an activation of tryptophan synthetase activity has been examined with germinated conidia. Table 4 shows that the indolyl compounds tested neither inhibit nor increase tryptophan synthetase activity. These results also indicate that these indole derivatives do not significantly affect the entry of indole into these cells.

TABLE 3

Effect of analogues and precursors of tryptophan on formation of tryptophan synthetase by strain 74A

Additions*	Germinated Conidia	Tryptophan Synthetase
	mg/ml	*specific activity*
None	3.4	109
L-Tryptophan	3.5	104
5-Methyl-DL-tryptophan	3.6	150
6-Methyl-DL-tryptophan	2.1	182
Indole	3.5	130
Indole-3-acetic acid	3.4	236
Anthranilic acid	3.3	128

* At 1.0 μmole per ml in germination medium.

TABLE 4

Effect of indolyl compounds on activity of tryptophan synthetase of strain 74A

Addition*	Tryptophan synthetase
	specific activity
None	95
L-Tryptophan	97
5-Methyl-DL-tryptophan	96
6-Methyl-DL-tryptophan	93
Indole-3-acetic acid	96

* At 1.0 μmole per ml in assay system.

Correspondence of indole uptake by whole cells and extracts. The possibility of obtaining cells of varying tryptophan synthetase activities afforded an opportunity to determine whether indole uptake by whole cells corresponded to the tryptophan synthetase activity of cell extracts. Table 5 gives the results of two such experiments. Here it can be seen that there is a close correspondence between whole cell and extract activities. In experiment 1, analyses for tryptophan were made on the reaction mixture after the extraction of indole; the tryptophan found was equivalent to 71 ± 3% of the indole which had disappeared. Thus, even discounting the likelihood of degradation of tryptophan by these crude extracts, the bulk of the indole which disappeared could be accounted for in terms of tryptophan synthetase activity. Experiment 2 shows that indolepropionic and indolebutyric acids also stimulate the formation of tryptophan synthetase activity, whereas tryptamine has no effect. It should be noted that the tryptophan synthetase activities in experiment 2 are somewhat lower than those usually obtained, but the pattern and extent of stimulation of formation of tryptophan synthetase activity is similar to that indicated in experiment 1 and Table 3; also, the ratios of extract to whole cell activities are similar.

A further comparison can be made between extract and whole cell activities of the same cells. When the activities of the extracts are calculated on the basis of the milligrams dry weight of cells extracted, the values obtained are within 15% of the same whole cell activities.[3] If it is assumed that all of the tryptophan synthetase activity was extracted, then it appears that there is no significant masking of tryptophan synthetase activity in whole cells. In this respect it might be noted that tryptophan synthetase can be extracted in high yield by gently shaking lyophilized mycelium in buffer (Suskind, 1957). The above results strongly indicate that indole uptake by whole cells is a valid measure of tryptophan synthetase activity.

Effect of tryptophan concentration on formation of tryptophan synthetase activity in a tryptophan auxotroph (strain 10575A). The preceding experiments have indicated that exogenous tryptophan does not repress, and certain analogues of tryptophan actually stimulate, the formation of tryptophan synthetase in strain 74A. But strain 74A is capable of synthesizing tryptophan and the possibility exists that there is always sufficient endogenous tryptophan present for a maximal repression of tryptophan synthetase formation. Thus, the addition of tryptophan to the germination medium would have no appreciable repressive effect. However, with a tryptophan auxotroph blocked in an early step in tryptophan synthesis some control over tryptophan levels in the cell could be achieved, and perhaps a repressive effect of tryptophan would be manifested. The reduction of intracellular repressor concentration by a variety of techniques has revealed repressible enzyme systems in other organisms (Vogel, 1961).

Conidia from strain 10575A were germinated in media containing various levels of tryptophan and the tryptophan synthetase activities determined. The results are given in Table 6. The growth of strain 10575A under these conditions is largely dependent on the concentration of tryptophan, and reaches a maximal value at 0.1 to 0.2 μmole tryptophan per ml. Some growth occurred in the absence of added tryptophan which might be due to an endogenous store of tryptophan, to the incompleteness of the genetic block (Bonner, Yanofsky, and Partridge, 1952), or to polysaccharide formation. However, visual examination of the cultures showed that most of the spores had germinated

[3] The extraction procedure gave an average yield of protein equivalent to 25.6 ± 3% of the dry weight of cells extracted; 1.0 mg of extract protein represents 3.9 mg dry wt cells. Since specific activities for whole cells are on a 1.0 hr basis and for extracts on a 3.0 hr basis, the whole cell equivalent of the latter is specific activity of extract × 3.0/3.9.

TABLE 5

Comparison of tryptophan synthetase activity of whole cells and extracts of strain 74A

Additions*	Experiment 1			Experiment 2		
	Tryptophan synthetase		Extract/ whole cell	Tryptophan synthetase		Extract/ whole cell
	Whole cells	Extract		Whole cells	Extract	
	specific activity			*specific activity*		
None	92	112	1.22	66	82	1.24
L-Tryptophan	83	103	1.24			
6-Methyl-DL-tryptophan	152	203	1.34			
Indole-3-acetic acid	198	259	1.31	124	143	1.15
Indole-3-propionic acid				144	172	1.20
Indole-3-butyric acid				95		
Tryptamine				65		
Indole				74	88	1.19
Anthranilic acid				90	111	1.23

* At 1.0 μmole per ml in germination medium, except 6-methyl-DL-tryptophan at 0.5 μmole per ml.

and an increased tryptophan synthetase activity was found, which would suggest that some protein synthesis occurred in the absence of exogenous tryptophan. Up to a level of 0.1 μmole per ml, the activity remains fairly constant, but between 0.1 and 0.2 μmole per ml, a sharp decrease occurs and the lower level of activity approximates that obtained with strain 74A. From about 1.0 to 2.0 μg tryptophan per ml, the activity shows a small but reproducible increase.

If it is assumed that there is an equivalence in cell composition at all levels of tryptophan, the data can be assessed on the basis of the relationship of the increase in activity to the increase in cell weight during germination. This assessment is shown in the last three columns of Table 6, where a 3-fold variation is observed in the activity of tryptophan synthetase. Thus, these results with strain 10575A indicate that tryptophan can probably repress the formation of tryptophan synthetase.

Effect of analogues of tryptophan on formation of tryptophan synthetase in strain 10575A. The increased formation of tryptophan synthetase by low level feeding of tryptophan appeared sufficient to determine the specificity of L-tryptophan as a repressor. Also, it was of interest to determine whether compounds which stimulated the formation of tryptophan synthetase in 74A would add to the effect of low level feeding of tryptophan. The results of such an experiment are given in Table 7. The growth of conidia was slightly inhibited by 4- and 6-methyltryptophan

TABLE 6

Effect of concentration of L-tryptophan on formation of tryptophan synthetase by strain 10575A

L-Tryptophan	Germinated Conidia	Tryptophan Synthetase		Increase*		
				Growth	Tryptophan synthetase	
μmoles/ml	*mg/ml*	*units/ml*	*specific activity*	*mg/ml*	*units/ml*	*specific activity*
0	1.39	281	202	0.69	233	323
0.02	1.82	342	188	1.12	294	262
0.05	2.78	573	206	2.08	525	252
0.10	3.09	581	188	2.39	533	223
0.20	3.32	359	108	2.62	311	118
0.50	3.28	341	104	2.58	293	113
1.0	3.20	336	105	2.50	288	115
1.5	3.22	364	113	2.52	316	125
2.0	3.18	394	124	2.48	346	139
Conidia	0.70	48	68			

* The values for the dry weight and activity of conidia were subtracted from similar values for germinated conidia.

and, because the control level of L-tryptophan was suboptimal, both indole and L-tryptophan afforded a small increase in growth; the other compounds had little effect on growth.

Tryptophan shows a high degree of specificity in reducing the formation of tryptophan synthetase, and only indole (which is converted to tryptophan) produced a comparable effect; 6-methyltryptophan also produces a significant inhibition, although this compound was stimula-

TABLE 7

Effect of analogues of tryptophan on formation of tryptophan synthetase by strain 10575A

Additions*	Germinated Conidia	Tryptophan Synthetase		Increase†		
				Growth	Tryptophan synthetase	
	mg/ml	*units/ml*	*specific activity*		*units/ml*	*specific activity*
None	2.90	632	218	2.02	572	283
L-Tryptophan	3.58	391	109	2.70	331	123
D-Tryptophan	3.08	650	212	2.20	590	268
4-Methyl-DL-tryptophan	2.45	534	218	1.57	474	302
5-Methyl-DL-tryptophan	3.03	672	222	2.15	612	285
6-Methyl-DL-tryptophan	2.65	443	167	1.77	383	216
Indole	3.34	401	120	2.46	341	139
Indole-3-acetic acid	2.80	622	222	1.92	562	292
Conidia	0.88	60	68			

* The medium contained 0.05 μmole L-tryptophan per ml and the listed compounds were added at a concentration of 0.5 μmole per ml.

† The values for the dry weight and activity of conidia were subtracted from similar values for germinated conidia.

tory in strain 74A. Indoleacetic acid and 5-methyltryptophan which stimulate tryptophan synthetase formation in strain 74A have no effect in strain 10575A. It might be noted that the levels of tryptophan synthetase activity achieved in strain 10575A approximates that obtained by strain 74A grown in the presence of indoleacetic acid. These results suggest that the stimulation of tryptophan synthetase formation by various analogues of tryptophan might depend on the presence of an intact system for tryptophan synthesis.

Repression and feedback inhibition prior to tryptophan synthetase. Although the action of tryptophan as a repressor of other steps in tryptophan synthesis is now being studied with respect to indole formation, some observations along these lines can be noted here. Strain 10575A accumulates anthranilic acid which can be readily spotted by its intense bluish fluorescence under ultraviolet light. It was observed that this fluorescence was most intense in cultures grown in media containing up to 0.1 μmole L-tryptophan per ml and almost nil at higher concentrations of tryptophan, suggesting either a strong repression or feedback inhibition in the biosynthetic sequence leading to anthranilic acid.

When germinated conidia of strain 10575A grown on low tryptophan (0.05 μmole per ml) were incubated in Fries medium containing 1.5% glucose, without tryptophan, a strong fluorescence appeared. If L-tryptophan, 4- or 6-methyltryptophan, or indole were added (1.0 μmole per ml), almost no fluorescence was observed, but the addition of 5-methyltryptophan or indoleacetic acid did not grossly affect the appearance of fluorescence. These observations suggest that tryptophan and certain tryptophan analogues markedly inhibit a reaction(s) involved in the synthesis of anthranilic acid.

Cells grown on a high supplement of tryptophan (0.5 μmole per ml) produced almost no fluorescence when incubated under the same conditions as indicated above. This could suggest a repressive action of tryptophan on the formation of an enzyme(s) concerned with the synthesis of anthranilic acid. But the failure of such cells to produce fluorescence could be due to an inhibition of anthranilic acid synthesis by tryptophan accumulated during germination; this possibility is being investigated.

DISCUSSION

The data presented indicate that the formation of tryptophan synthetase in *N. crassa* is probably subject to repression by tryptophan. However, a repressive action of tryptophan was evidenced only by the presumed release of tryptophan synthetase formation, achieved by germination of conidia of a tryptophan dependent strain, 10575A, in the presence of concentrations of

tryptophan which were suboptimal for growth. The lack of a repressive effect of added tryptophan in a tryptophan independent strain, 74A, suggested the possibility that endogenous levels of tryptophan in strain 74A were sufficient to afford a maximal repression of tryptophan synthetase. A cursory assessment of endogenous tryptophan, in trichloroacetic acid extracts of conidia and germinated conidia of strain 74A, has indicated the presence of 0.3 to 1.0 μmole tryptophan per ml of cell water. It might be noted that similar levels of added tryptophan produced a maximal repression in strain 10575A (Table 6), and in the formation of tryptophan synthetase by cell-free extracts of Neurospora (Wainwright, 1959). The action of L-tryptophan appears to be quite specific since only indole and, to a lesser extent, 6-methyltryptophan repressed tryptophan synthetase formation; D-tryptophan, 4- and 5-methyltryptophan, and indoleacetic acid had no effect.

The observation that certain analogues or precursors of tryptophan stimulated tryptophan synthetase formation in strain 74A, but not in strain 10575A, suggests that an intact tryptophan-synthesizing system was necessary for the stimulatory effects of these compounds. Some possible explanations for this effect are that these compounds reduce the levels of endogenously produced tryptophan by (i) inhibiting some step in tryptophan synthesis, (ii) inducing increased tryptophan metabolism, or (iii) by competing with tryptophan for a concentrating system. The first possibility would appear to be supported by the observation of an inhibition of anthranilic acid formation by 6-methyltryptophan. However, neither 5-methyltryptophan nor indoleacetic acid appeared to inhibit anthranilic acid formation, nor do they inhibit indole synthesis (*unpublished data*), but both, and especially indoleacetic acid, stimulate tryptophan synthetase formation in strain 74A. Since there is no apparent correlation between an inhibition of early steps in tryptophan synthesis and stimulatory activity, and none of the stimulatory compounds affect tryptophan synthetase activity, it is unlikely that their stimulation of tryptophan synthetase formation in strain 74A is due to an inhibition of tryptophan synthesis. The other possibilities are being investigated.

The present experiments have shown that approximately a 3-fold increase in tryptophan synthetase activities can be achieved by germinating strain 10575A in the presence of low levels of tryptophan, or in strain 74A by adding indoleacetic acid to the germination medium. This range is quite small by comparison to that obtained with tryptophan synthetase in bacteria (Monod and Cohen-Bazire, 1953; Lester and Yanofsky, 1961), and raises the question of whether the variation in tryptophan synthetase formation observed here represents the actual limits of repression and release. In this respect it can be noted that similar variations of tryptophan synthetase formation have been observed in older cultures (Yanofsky, 1955; Suskind, *personal communication*) and in cell-free extracts (Wainwright, 1959) as a function of tryptophan concentration. Also, in strain 10575A, the lowest level of tryptophan synthetase is obtained with about 0.2 μmole L-tryptophan per ml, and this level is not lowered further by a 5-fold increase in tryptophan. Thus, it appears that the level of tryptophan synthetase in strain 10575A germinated in the presence of excess tryptophan, as well as the similar levels in strain 74A, represent minimal values for tryptophan synthetase in germinated conidia. Whether the upper limits of tryptophan synthetase formation observed are maximal is not known. However, the aforementioned observations on the variation in tryptophan synthetase formation in whole cells and extracts would suggest that much further release than that observed here is unlikely. It is of interest to note that proline has a similarly small effect on the level of pyrroline-5-carboxylate reductase in Neurospora (Yura and Vogel, 1959).

In bacteria repressibility is a genetic trait, and mutants have been obtained in which the formation of certain enzymes such as β-galactosidase (Pardee, Jacob, and Monod, 1959), ornithine transcarbamylase (Maas, 1960), or tryptophan synthetase (Cohen and Jacob, 1959) is no longer responsive to the corresponding repressors. This raises the possibility that the strains of Neurospora examined here are the equivalent of nearly nonrepressible mutants, with respect to tryptophan synthetase formation. The tryptophan synthetase activity of many strains of Neurospora has been determined (Yanofsky; Suskind; *personal communications*) and no greater variation has been observed than was obtained with either of the strains examined in this study. The negation of this possibility would depend on the discovery of a more repressible strain, as appears to have been found in

the case of arginine synthesis in *Escherichia coli* (Gorini; *see* Vogel, 1961) or achieving a greater release than that observed here.

The present studies do suggest that the tryptophan synthetase step in Neurospora is not the point for an effective regulation of tryptophan synthesis. As indicated by the observations on anthranilic acid formation, a feedback inhibition and repressor action by tryptophan might be very pronounced on enzymes preceding tryptophan synthetase in the biosynthesis of tryptophan. This would suggest that if coordinate repression (Ames and Garry, 1959) does occur it does not significantly include the final enzyme in the sequence of enzymes leading to tryptophan formation. This possibility is now being investigated.

SUMMARY

The formation of tryptophan synthetase in Neurospora has been examined in germinated conidia from two strains. In a tryptophan independent strain no effect of tryptophan was observed, but certain analogues of tryptophan and especially indoleacetic acid produced a 2- to 3-fold stimulation of tryptophan synthetase formation. In a tryptophan dependent strain, the level of tryptophan synthetase was inversely dependent on the concentration of tryptophan in the germination medium, but the lowest and highest activities obtained differed only by a factor of three. Tryptophan appears to have a marked inhibitory effect on the formation and activity of an anthranilic acid synthesizing system in the tryptophan dependent strain.

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