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Regulation of Tryptophan Biosynthetic Enzymes in *Neurospora crassa*

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The formation of enzymatic activities involved in the biosynthesis of tryptophan in *Neurospora crassa* was examined under various conditions in several strains. With growth-limiting tryptophan, the formation of four enzymatic activities, anthranilic acid synthetase (AAS), anthranilate-5-phosphoribosylpyrophosphate phosphoribosyl transferase (PRAT), indoleglycerol phosphate synthetase (InGPS), and tryptophan synthetase (TS) did not occur coordinately. AAS and TS activities began to increase immediately, whereas PRAT and InGPS activities began to increase only after 6 to 12 hr of incubation. In the presence of amitrole (3-amino-1,2,4-triazole), the formation of TS activity in a wild-type strain was more greatly enhanced than were AAS and InGPS activities. With a *tr-3* mutant, which ordinarily exhibits an elevated TS activity, amitrole did not produce an increase in TS activity greater than that observed on limiting tryptophan. With *tr-3* mutants, the increased levels of TS activity could be correlated with the accumulation of indoleglycerol in the medium; prior genetic blocks which prevented or reduced the synthesis of indoleglycerol also reduced the formation of TS activity. The addition of indoleglycerol to cultures of a double mutant (*tr-1*, *tr-3*) which could not synthesize indoleglycerol markedly stimulated the production of TS activity but not PRAT activity; the production of TS activity reached the same level with limiting or with excess tryptophan. A model explaining these and other related observations on enzyme formation in *N. crassa* is proposed.

The sequence of reactions specific for the biosynthesis of tryptophan in *Neurospora crassa* is now well established, in enzymatic and genetic terms (Table 1). As clear an understanding of the regulation of this system has not been achieved, although it appears that the tryptophan pathway may be subject to a variety of controls. The occurrence of regulation at the level of enzyme activity has been unequivocally demonstrated; tryptophan can exert a negative feedback control through its ability to inhibit anthranilic acid synthetase (AAS) and phosphoribosyl-anthranilate transferase (PRAT), as demonstrated in vitro (8) and in vivo (17, 18). At the level of enzyme formation several regulatory phenomena have been reported, but precise explanations of these phenomena are yet to be proposed or verified.

In cells obtained from cultures with growth-limiting amounts of tryptophan, the activity of tryptophan-synthesizing enzymes is about two to five times as high as in cells grown in the presence of an excess of tryptophan, suggesting that tryptophan can repress the formation of these enzymes (16–18, 20). In vivo studies (17, 18) indicate that the formation of tryptophan enzymes is not coordinately controlled, as are similar enzymes in *Escherichia coli*, which are specified by closely linked genes (22, 31). In histidine auxotrophs of *Neurospora*, a limitation of histidine can produce a derepression of the formation of tryptophan enzymes (6), and it appears that the formation of these enzymes occurs coordinately or in parallel (5). The histidine effect is not related to the availability of free histidine, externally or internally, but, more likely (M. Carsiotis and G. Lester, Bacteriol. Proc., p. 136, 1968), to a depletion of charged histidinyl-transfer ribonucleic acid (tRNA). It has also been observed (24) that amitrole (3-amino-1,2,4-triazole) and indoleacrylic acid elicit the production of imidazoleglycerol (phosphate) and indoleglycerol (phosphate), respectively, with a coincident increased formation of tryptophan synthetase (TS). These results could not be attributed to a depletion of tryptophan, and it could be concluded (24) that imidazoleglycerol (phosphate) and indoleglycerol (phosphate) were acting as inducers of TS. Amitrole also interferes with histidine biosynthesis and inhibits the induction of kynureninase formation (24).

TABLE 1. *Reactions, enzymes, and genetic determinants of the tryptophan biosynthetic pathway in Neurospora*[a]

Reaction	Enzyme	Genetic locus[b]
1. Chorismic acid → anthranilic acid	AAS	*tr-2*
2. Anthranilic acid → PRA	PRAT	*tr-4*
3a. PRA → CDRP	PRAI	*tr-1*
3b. CDRP → InGP	InGPS	*tr-1*
4a. InGP + serine → tryptophan	TS	*tr-3*
4b. InGP → Indole + glycerol phosphate	TS	*tr-3*
4c. Indole + serine → tryptophan	TS	*tr-3*

[a] Abbreviations: PRA, *N*-(5'-phosphoribosyl)anthranilate; CDRP, 1-(*O*-carboxyphenylamino)-1-deoxyribulose 5-phosphate; InGP, indole-3-glycerol phosphate; AAS, anthranilic acid synthetase; PRAT, anthranilate-5-phosphoribosylpyrophosphate phosphoribosyl transferase; PRAI, PRA isomerase; InGPS, INGP synthetase; TS, tryptophan synthetase.

[b] The enzymatic activities determined by the *tr-1* and *tr-2* loci occur as a tightly bound multienzyme aggregate (11). Mutations of the *tr-1* gene may cause a loss of any one or a combination of AAS, PRAI, and InGPS activities; mutations of the *tr-2* gene usually affect only AAS activity (10). Mutations of the *tr-3* gene are usually observed as defects in reactions 4a and 4c, 4a and 4b, or in 4a, b, and c (9).

The present study examines more directly the question of the parallel formation of tryptophan enzymes under conditions of tryptophan limitation and the ability of indoleglycerol (phosphate) to stimulate the formation of tryptophan enzymes other than TS. It is shown that the formation of tryptophan-synthesizing enzymes does not occur in parallel and that indoleglycerol (phosphate) appears to specifically induce the formation of only TS.

MATERIALS AND METHODS

Organisms. The *Neurospora* strains used in this study and their enzymatic defects are listed in Table 2. Double mutants were produced by standard crossing procedures, and the progeny ascospores were randomly isolated.

Culture conditions. Vogel medium N (26) was used as the basal medium. Still cultures were grown in medium N containing 2% sucrose and supplements as indicated; the inoculum was 2×10^4 to 4×10^{-4} conidia per ml, and cultures were incubated at 30 C for 68 to 72 hr unless otherwise noted. Shake cultures of germinated conidia were grown in medium N containing 1% sucrose and supplements indicated. The inoculum was about 2×10^7 conidia per ml, and the cultures were incubated at 30 C for 17 to 18 hr with vigorous agitation on a rotary shaker. Cultures were harvested by filtration, and the filtrates were saved for appropriate analyses. The mycelia were rinsed thoroughly with distilled water and lyophilized, and the dry weight was determined. (Deviations from this procedure will be noted.)

Enzyme assays. Lyophilized mycelia were pulverized by the method of Fankhauser (cf. 6) and extracted with 0.05 M potassium phosphate buffer (*p*H 7.0) containing 10^{-4} M ethylenediaminetetraacetate. In two experiments (Table 3 and Fig. 1B), wet cells were ground in a Ten Broeck mill and extracted with 0.02 M Tris buffer (*p*H 7.5) containing 0.5 μmole of glutathione and 4 μg of pyridoxal phosphate per ml; dry weights were determined from oven-dried samples of the germinated conidia cultures. In all cases, 25 ml of extractant was used per 1.0 g (dry weight) of cells. Cell-free extracts were obtained by centrifugation at 27,000 × *g* for 30 min. In some instances, these extracts were passed over G-25 Sephadex to remove small molecules, but comparisons of enzyme activities in Sephadex-treated and untreated extracts showed little difference (<20%).

AAS activity was estimated from the formation of anthranilic acid from chorismic acid (8). Assays of PRAT and indoleglycerol phosphate synthetase (InGPS) activities were based on the procedures of Wegman and DeMoss (28). TS activity (Table 1, reaction 4c) was determined by the method of Yanofsky (29). Phosphoribosyl anthranilate isomerase (PRAI) activity was not measured. Enzyme activity assays were carried out at 35 C, usually for a 30-min period. Enzyme specific activity is designated as nanomoles of substrate used or product formed per minute per milligram of protein.

Other assays. Tryptophan was estimated from the

TABLE 2. *Genetic and enzymatic identification of Neurospora strains*

Strain no.[a]	Mutated locus	Defective enzymes[b]
74A	*wt*	None
10575A	*tr-1*	InGPS
Ind-II-13A	*tr-1*	AAS, InGPS
RC-5-1A	*tr-2,3*	AAS, TS (4a, c)
Td-141A	*tr-3*	TS (4a, b)
A-78a	*tr-3*	TS (4a, b)
Ind-I-9A	*tr-4*	PRAT
RC-12-1A	*tr-4*	PRAT
RC-25-1A	*tr-4*	PRAT
RC-31-1A	*tr-2,3*	AAS, TS (4a, b)
RC-31-2A	*tr-3*	TS (4a, b)
RC-31-3A	*tr-2*	AAS
RC-31-4A	*wt*	None
RC-33-1A	*tr-1,3*	AAS, InGPS, TS (4a, b)
RC-33-2A	*tr-3*	TS (4a, b)
RC-33-3A	*tr-1*	AAS, InGPS
RC-33-4a	*wt*	None

[a] RC strains were produced in this laboratory; in RC-12, 25, 31, and 33, the *tr-1,2,4* mutations were derived from strain 74A by treatment with ICR-170. Each of the RC-31, -33 series was obtained from the progeny of a single cross; the *tr-3* locus in each case is Td-141A. All other strains were obtained from the Bonner collection (University of California, San Diego; cf. J. A. DeMoss).

[b] See Table 1 for abbreviations.

indole produced upon treatment of samples with tryptophanase (12). Indoleglycerol was determined as the indole-3-aldehyde formed by periodate oxidation of samples (30). Anthranilic acid was extracted from acidified (*p*H 4.0) samples with ethyl acetate and measured by fluorimetry, with excitation at 340 nm and emission at 400 nm, in an Aminco-Bowman spectrophotofluorimeter. Protein was measured by the method of Lowry et al. (19).

Preparations. Chorismic acid was isolated from cultures of *Aerobacter aerogenes*, 62-1 (obtained from J. A. DeMoss) by the procedures of Gibson (14). 1-(*O*-carboxyphenylamino)-1-deoxyribulose 5-phosphate was prepared chemically by the method of Smith and Yanofsky (23).

Indoleglycerol was isolated from cultures of *E. coli* strain T-26 (obtained from C. Yanofsky); a typical procedure is as follows. Strain T-26 is grown in 1 liter of half-strength Vogel medium E (27) containing 0.3% glucose (autoclaved separately) and 5 μg of L-tryptophan per ml; the culture is incubated at 30 C for 20 hr on a rotary shaker. The cells are removed by centrifugation, resuspended in fresh medium without tryptophan, similarly incubated for 10 to 20 hr, and centrifuged. The combined culture supernatant fluids are adjusted to *p*H 7.0 with sodium hydroxide; periodate assays indicate 0.7 to 1.2 μmoles of indoleglycerol per ml. With constant stirring, 3 to 4 g of Norit A is added in 0.5 to 1.0-g portions until the supernatant fluids of centrifuged samples show little indoleglycerol remaining. A convenient spot test for indoleglycerol can be made by the addition of 1.5 volumes of Salkowski's reagent (15) to the samples. The Norit is collected by filtration on a Büchner funnel and rinsed with 25 ml of water. The Norit is eluted with 500 to 600 ml of 50% ethanol in 100-ml batches, with stirring and filtering, until the eluates show little indoleglycerol. The combined eluates are reduced to a volume of 15 ml under vacuum at 40 C with a rotary evaporator. At this point, a recovery of 50 to 60% of the starting indoleglycerol, with a purity of about 65%, may be expected. The concentrated eluate is chromatographed with G-10 Sephadex; 10 to 15 ml is placed on a column (2.5 by 34 cm) and eluted with very dilute saline (5 mg of NaCl/liter) at a rate of 0.5 to 0.8 ml/min. Fractions of 10 ml are collected and spot tested; indoleglycerol is first observed in about fraction 40, representing the prior elution of about five times the liquid volume of the column, which suggests that indoleglycerol is strongly adsorbed to G-10 Sephadex. Most of the indoleglycerol appears in fractions 40 to 52; these are combined and reduced to a volume of 30 ml under vacuum at 40 to 45 C. This product contains almost all of the indoleglycerol placed on the column and exhibits a purity close to 100%. The absorption spectrum of periodate-oxidized material is identical to that of indole-3-aldehyde. It may be noted that indole-3-glycerol phosphate is not adsorbed to G-10 Sephadex, and, with the above conditions, it is eluted with a little more than one liquid volume of the column.

RESULTS

Effect of tryptophan levels on growth and enzymatic activities. Germinated conidia of a wild type and several tryptophan auxotrophs were obtained from cultures containing growth-limiting and excess tryptophan, and their enzymatic activities were determined (Table 3). Aside from strain 74A and strain 10575A, a marked dependence on tryptophan for growth was observed; the nearly maximal growth of strain 10575A on the lower level of tryptophan could be attributed, in part, to the incompleteness of the genetic block (4).

The levels of activities from strain 74A do not appear to be influenced by tryptophan, and it might be assumed that the tryptophan formed endogenously is sufficient to maximally repress the formation of all the tryptophan enzymes. The relatively small differences in the specific activities of enzymes from strain 10575A grown on low and high levels of tryptophan might also be ascribed to the leakiness of this strain. AAS and InGPS activities differed among strains, both in absolute activity and in the degree of repression by tryptophan. However, with any one strain, the ratios of these activities in extracts from cells grown on low and high levels of tryptophan were very similar. The level of PRAT activity usually shows the same relative response to tryptophan concentrations as AAS and InGPS activities, but occasional differences have been observed, as with strain RC-5-1A. The influence of tryptophan levels in the medium on TS activity in tryptophan auxotrophs is usually quite different from that on AAS, PRAT, and InGPS activities. The data in Table 3 could indicate that, although the formation of AAS and InGPS activities might be coordinately influenced by tryptophan, this coordination is less apparent relative to the formation of PRAT and, especially, TS activities. It should be noted that strains carrying a mutant *tr-3* allele exhibit somewhat higher AAS and InGPS activities and considerably higher TS activity than other strains when grown on limiting tryptophan. When grown on excess tryptophan, the mutant *tr-3* strains show a heightened activity only in the case of TS. Since the mutant *tr-3* strains accumulate indoleglycerol, the enhanced activities may be due to an induction, especially of TS activity, by indoleglycerol (phosphate) (24).

Depression of enzyme formation during growth. Closer examinations of the formation of enzymatic activities concerned with tryptophan biosynthesis were made during the growth of cultures on limiting tryptophan. Conidia were germinated under conditions of maximal repression (0.5 μmole of L-tryptophan per ml) and were then transferred to a medium with a relatively low level of tryptophan (0.2 μmole/ml). Samples were taken at various times, and the cells were

TABLE 3. *Influence of tryptophan on growth and on the levels of enzymatic activities involved in tryptophan biosynthesis*[a]

Strain (locus)	Culture		Specific activity[b]			
	L-Tryptophan (μmole/ml)	Dry wt (mg/ml)	AAS	PRAT	InGPS	TS
74A (*wt*)	0.1	5.2	0.67	1.50	1.83	1.83
	0.5	5.9	0.83	1.50	1.67	2.00
			(0.8)	(1.0)	(1.1)	(0.9)
10575A (*tr-1*)	0.1	4.1	1.67	2.50	nil	3.82
	0.5	5.3	0.83	1.17	nil	1.83
			(2.0)	(2.1)		(2.1)
Ind II-13A (*tr-1*)	0.1	2.5	nil	7.81	nil	7.15
	0.5	6.1	nil	1.83	nil	2.16
				(4.3)		(3.3)
RC-5-1A (*tr-2,3*)	0.1	2.5	nil	8.15	7.15	nil
	0.5	5.1	nil	2.33	1.50	nil
				(3.5)	(4.8)	
Td-141A (*tr-3*)	0.1	2.2	4.48	8.30	11.3	30.7
	0.5	5.3	1.17	2.00	2.66	15.2
			(3.9)	(4.4)	(3.9)	(2.0)
A-78a (*tr-3*)	0.1	1.9	4.65	5.32	9.50	20.6
	0.5	5.2	1.17	1.17	2.50	6.82
			(4.0)	(4.5)	(3.8)	(3.0)
Ind I-9A (*tr-4*)	0.1	2.1	2.66	nil	3.00	8.00
	0.5	4.9	1.00	nil	1.33	2.33
			(2.7)		(2.3)	(3.4)

[a] Values given are the averages of two experiments; the values obtained in replicate experiments did not differ from their average value by more than 7%.

[b] Specific activity values are nanomoles per minute per milligram of protein. Numbers in parentheses are the ratios of activities in extracts from cells grown on 0.1 μmole to those grown on 0.5 μmole of tryptophan per ml. Nil represents a specific activity of less than 0.20. See Table 1 for abbreviations.

extracted and analyzed for enzymatic activities and for tryptophan; the culture filtrates were analyzed for anthranilate, indoleglycerol, and tryptophan. Growth was estimated in terms of cell dry weight.

The results of a typical experiment with strain Td-141A are illustrated in Fig. 1. The tryptophan level in the medium fell rapidly. Intracellular tryptophan decreased less rapidly, but by 8 to 10 hr the cells were almost devoid of tryptophan. Anthranilate and indoleglycerol concentrations in the medium remained quite low until intracellular tryptophan disappeared; they then increased rapidly. This was consistent with the earlier mentioned inhibitory action of tryptophan on AAS and PRAT activities. The difference in the times at which anthranilate and indoleglycerol began to increase might have been due to the fact that the PRAT and InGPS activities were initially higher than AAS activity, and much of the anthranilate formed shortly after the exhaustion of tryptophan could have been converted to indoleglycerol.

Figure 1B shows a marked difference in the relative rate of formation of enzymatic activities. AAS and TS activities began to increase almost immediately, whereas PRAT and InGPS activities did not begin to rise until after 12 hr of incubation. Similar experiments were run with strains Td-141A and Ind-II-13A (on indole and tryptophan) and with strain Ind-I-9A (on tryptophan). The same pattern was observed in all cases; AAS and TS activities began to increase almost immediately, whereas PRAT and InGPS activities began to increase only after 6 to 12 hr of incubation. These observations indicate that the enzymes involved in tryptophan biosynthesis are not formed coordinately.

Influence of amitrole on the formation of enzymatic activities. The stimulation of the formation of TS activity by amitrole has been attributed (5, 24) to the concomitant limitation of histidine, the

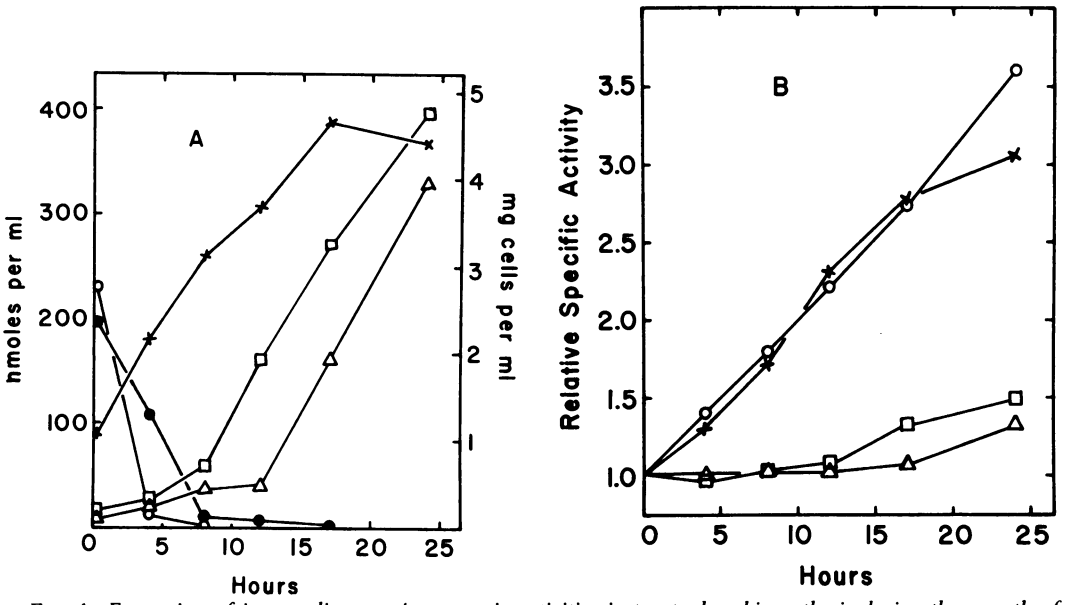

FIG. 1. *Formation of intermediates and enzymatic activities in tryptophan biosynthesis during the growth of strain Td-141A (tr-3) on limiting tryptophan. A. Anthranilate* (△), *indoleglycerol* (□), *and tryptophan* (○) *in culture filtrates; tryptophan in cell extracts* (●); *cell dry weight* (×). *B. Specific activity values for each enzyme are normalized to a 0-hr value of 1.0; the actual specific activities (nanomoles per minute per milligram of protein) at 0 hr are: AAS* (○), *1.00; PRAT* (△), *2.33; InGPS* (□), *2.50; TS* (×), *11.7.*

accumulation of imidazoleglycerol (phosphate), or both. Thus, it was of interest to examine the effect of amitrole on the formation of other enzymatic activities in tryptophan biosynthesis and to determine whether the amitrole effect was influenced by tryptophan.

Germinated conidia of strain 74A were produced in cultures containing various levels of amitrole. Growth was estimated as cell dry weight; culture filtrates were analyzed for indoleglycerol; and cell extracts were assayed for protein, TS, InGPS, and AAS activities. The data in Table 4 indicate that amitrole did not affect the growth of this strain except at the highest concentration used, where, presumably, the limitation of histidine synthesis was sufficient to inhibit growth. No indoleglycerol was detected in the culture filtrates. The specific activity of each enzyme progressively increased with increasing levels of amitrole, but this increase was much more dramatic in the case of TS than that of InGPS and AAS. These results are generally similar to those previously reported (24; and R. Jones and M. Carsiotis, Bacteriol. Proc., p. 114, 1967). However, the increased activities obtained in the absence of an inhibition of growth might suggest that imidazole-glycerol (24) might be of greater significance in stimulating the formation of these enzymes than a growth-limiting depletion of histidine. This is particularly notable in the case of TS activity and indicates that the formation of TS activity can be disproportionately stimulated relative to the other two enzymatic activities.

The influence of tryptophan on the effect of amitrole and on the accumulation of indoleglycerol, relative to the level of enzymatic activities, was examined in germinated conidia cultures of strain Td-141A (*tr-3*) which accumulates indoleglycerol and still retains the indole + serine → tryptophan activity of TS. The results given in Table 5 show that with growth-limiting tryptophan (0.1 μmole/ml) more indoleglycerol accu-

TABLE 4. *Effect of amitrole on the formation of TS, InGPS, and AAS activities by strain 74A (wt)*

Culture			Specific activity[a]		
Amitrole (μmole/ml)	Dry wt (mg/ml)	Indoleglycerol (μmole/ml)	TS	InGPS	AAS
0	5.4	<0.01	1.83	1.83	1.00
1.0	5.6	<0.01	4.50 (2.8)	2.33 (1.3)	1.17 (1.2)
2.0	5.6	<0.01	8.00 (4.4)	3.00 (1.6)	1.50 (1.5)
3.0	3.6	<0.01	18.7 (10)	4.17 (2.3)	2.67 (2.7)

[a] Specific activity values are nanomoles per minute per milligram of protein. Numbers in parentheses are the specific activity values normalized to those with no amitrole as 1.0. See Table 1 for abbreviations.

TABLE 5. *Effect of amitrole on the formation of TS, InGPS, and AAS activities by strain Td 141A (tr-3)*

Expt no.	Culture				Specific activity[a]		
	L-Tryptophan (μmole/ml)	Amitrole (μmoles/ml)	Dry wt (mg/ml)	Indoleglycerol (μmole/ml)	TS	InGPS	AAS
1	0.1	0	1.5	0.45	34.5	5.50	4.67
	0.5	0	5.3	0.20	17.5	1.83	1.33
	0.5	0.5	4.5	0.40	31.0	2.84	3.17
	0.5	1.0	3.7	0.37	37.2	3.50	3.84
	0.5	1.5	3.2	0.31	39.2	4.17	3.33
2	0.1	0	1.6	0.34	34.5	8.00	4.50
	0.1	1.5	1.1	0.35	40.0	8.50	5.00
	0.5	0	5.5	0.25	21.0	3.50	2.00
	0.5	1.5	2.8	0.31	39.6	6.17	3.67

[a] Specific activity values are nanomoles per minute per milligram of protein. See Table 1 for abbreviations.

mulates than with excess tryptophan (0.5 μmole/ml). Correspondingly, enzyme activities are higher in extracts from cells grown on the lower level of tryptophan. In the absence of amitrole, the direct relationship of enzymatic activity to indoleglycerol is most pronounced in the case of TS activity which is 10 to 20 times higher than TS activity in strain 74A. The activities of InGPS and AAS from cells grown on the high level of tryptophan are not much greater than those observed in strain 74A, and on the lower level of tryptophan these activities are no more than twice as high as those observed in situations where indoleglycerol does not accumulate (e.g., Table 4, and strain Ind-I-9A in Table 3). Thus, it appears that the stimulatory or inductive effect of indoleglycerol is primarily directed towards the formation of TS activity.

The ability of amitrole to overcome the repressive effect of tryptophan is shown in experiment 1 of Table 5. In the presence of excess tryptophan, increasing amounts of amitrole elicit a progressive decrease in growth and an increase in enzymatic activities, amounting, maximally, to about 2.5 times as high an activity as in the absence of amitrole, or about the same levels of activity observed on growth-limiting tryptophan alone. Experiment 2 of Table 5 examined the ability of amitrole to influence the formation of enzymatic activities in the presence of growth-limiting and excess levels of tryptophan. Here, with limiting tryptophan, amitrole caused only a 15% increase in TS activity and no significant change in the activities of InGPS and AAS. Thus, with the maximal derepression obtained with a limitation of tryptophan, amitrole had no significant additive effect.

Influence of indoleglycerol (phosphate) on the formation of enzymatic activities. The experiments described above are consonant with those previously reported (24), which ascribe to indoleglycerol phosphate (or imidazoleglycerol phosphate) an inducer-like role in the formation of TS activity. This conclusion was largely derived from work with inhibitors such as amitrole and indole acrylic acid (24; and J. R. Turner and W. H. Matchett, Bacteriol. Proc., p. 144, 1970). Also, the influence of indoleglycerol (phosphate) on the formation of enzymatic activities other than TS activity has not been determined. The following series of experiments examined the function of indoleglycerol (phosphate) under more normal physiological conditions and, more directly, its effect on the formation of several enzymatic activities. Use was made of various tryptophan auxotrophs which may or may not accumulate indoleglycerol under certain conditions, and the effect of indoleglycerol added directly to cultures was examined. All of the following experiments were performed with still cultures, prepared as described above.

The putative effect of indoleglycerol phosphate on the formation of TS activity was examined with a group of strains derived from the same cross, *tr-1* × *tr-3*. The *tr-1* strain lacked AAS, PRAI, and InGPS activities; the *tr-3* strain lacked the 4a and 4b activities of TS (see Table 1) and could carry out tryptophan biosynthesis to indoleglycerol phosphate which is usually excreted as indoleglycerol. Table 6 shows that indoleglycerol is accumulated only in cultures of strain RC-33-2A (*tr-3*) and that the TS activity from strain RC-33-2A is much higher than that obtained from the other strains. It is notable that an enhancement of TS activity is observed even with a very low accumulation of indoleglycerol. The data for strain RC-33-1A (*tr-1*,*tr-3*) also show that high-level formation of TS activity is not a peculiar characteristic of this *tr-3* allele.

The correlation of indoleglycerol accumulation and the formation of several enzymatic activities was examined with a group of strains derived

from a cross between *tr-2* and *tr-3* strains. The *tr-2* strain lacked AAS activity, and the *tr-3* strain was the same as the one described above. The ditype strain *tr-2,tr-3* still retained the reactions of the tryptophan pathway which convert anthranilic acid to indoleglycerol phosphate. With this ditype, the level of indoleglycerol may be directly influenced by the omission or addition of anthranilic acid to the medium (18). Table 7 shows that indoleglycerol accumulated only in cultures of strain RC-31-2A (*tr-3*) and strain RC-31-1A (*tr-2, tr-3*). In the latter case, the production of indoleglycerol in the absence of added anthranilic acid was probably due to the conversion of tryptophan to anthranilate by way of the tryptophan-anthranilate cycle (21). In the presence of the higher level of tryptophan, indoleglycerol accumulation was low even in the presence of anthranilic acid, since tryptophan inhibits the activity of AAS and PRAT (8, 18). The occurrence of indoleglycerol was paralleled by increased TS activity; this relationship is particularly apparent with strain RC-31-1A where the addition of anthranilic acid increased the accumulation of indoleglycerol and the level of TS activity to that observed with strain RC-31-2A. The effect of indoleglycerol (phosphate), as was the case with amitrole, was most pronounced with TS activity, which was brought to a level about 20 times that found in the strains which did not accumulate indoleglycerol. The increase in other enzymes was, at best, only about five times that obtained from strains RC-31-3A and RC-31-4A. These results indicate that indoleglycerol (phosphate) can markedly stimulate the formation of TS activity. The experiment described in Table 7 and the results obtained are analagous to those reported by Crawford and

TABLE 6. *Influence of a prior block (tr-1) in tryptophan biosynthesis on the formation of TS activity by a tr-3 mutant strain grown in still culture*

Strain (locus)	Culture			TS (specific activity)[a]
	L-Tryptophan (μmole/ml)	Dry wt (mg/ml)	Indoleglycerol (μmole/ml)	
RC-33-2A (*tr-3*)	0.15	1.4	0.40	40.1
	0.6	2.1	0.04	10.0
RC-33-1A (*tr-1,3*)	0.15	0.9	<0.01	4.83
	0.6	2.5	<0.01	1.67
RC-33-3A (*tr-1*)	0.15	1.0	<0.01	5.50
	0.6	2.3	<0.01	2.50
RC-33-4a (*wt*)	0.15	2.0	<0.01	1.50
	0.6	2.5	<0.01	1.83

[a] Specific activity values are nanomoles per minute per milligram of protein.

TABLE 7. *Influence of a prior block (tr-2) in tryptophan biosynthesis on the formation of tryptophan enzymes by a tr-3 strain grown in still culture*

Strain (locus)	Culture				Specific activity[a]			
	L-Tryptophan (μmole/ml)	Anthranilic acid (μmole/ml)	Dry wt (mg/ml)	Indoleglycerol (μmole/ml)	TS	InGPS	PRAT	AAS
RC-31-2A (*tr-3*)	0.15	0	1.3	0.27	36.0	5.33	9.00	3.17
	0.15	0.35	1.1	0.23	36.8	4.50	7.83	2.33
	0.5	0	2.6	0.16	9.50	1.50	2.00	1.67
	0.5	0.35	2.1	0.11	8.17	1.83	2.00	1.00
RC-31-1A (*tr-2,3*)	0.15	0	1.0	0.05	11.7	4.33	8.50	nil
	0.15	0.35	1.1	0.27	35.7	4.83	8.50	nil
	0.5	0	2.5	0.04	7.00	1.33	2.00	nil
	0.5	0.35	1.7	0.05	11.0	1.83	2.33	nil
RC-31-3A (*tr-2*)	0.15	0	2.6	<0.01	4.67	1.67	3.67	nil
	0.15	0.35	2.3	<0.01	4.17	1.33	1.83	nil
	0.5	0	3.0	<0.01	2.33	0.67	1.83	nil
	0.5	0.35	2.1	<0.01	2.17	0.50	1.83	nil
RD-31-4A (*wt*)	0.15	0	3.0	<0.01	2.17	1.00	1.83	0.83
	0.15	0.35	2.7	<0.01	2.33	0.50	1.83	0.83
	0.5	0	3.1	<0.01	1.83	0.67	1.67	0.83
	0.5	0.35	2.7	<0.01	2.00	0.50	2.00	0.67

[a] Specific activity values are nanomoles per minute per milligram of protein. Nil represents a specific activity of less than 0.20.

Gunsalus (7), who showed that indoleglycerol phosphate can induce very high levels of tryptophan synthetase in *Pseudomonas putida*.

The previous experiments demonstrate that indoleglycerol (phosphate) formed in vivo can dramatically enhance the formation of TS activity. It was desirable to demonstrate, then, that a source of indoleglycerol (phosphate) less dependent on internal synthesis could elicit an increased formation of TS activity. Indoleglycerol was isolated as described in Materials and Methods and was used as a supplement (filter-sterilized) for media containing growth-limiting and excess levels of tryptophan. The test strain was RC-33-1A, which is devoid of AAS, PRAI, and InGPS activities and thus cannot synthesize indoleglycerol phosphate. The culture fluids were analyzed for indoleglycerol after incubation, and cell extracts were assayed for TS and PRAT activities. The data presented in Table 8 show that the levels of indoleglycerol in the medium at the end of the incubation period (71 hr) are about the same as those added initially. TS activity increases in parallel with the increased levels of indoleglycerol, whereas PRAT activity is not affected by the presence of indoleglycerol. The apparent full recovery of indoleglycerol added might suggest that it is indoleglycerol per se which effects the stimulation of formation of TS activity. However, the highest levels of TS activity observed in this experiment were about the same as those found previously when the concentration of indoleglycerol accumulated was about 0.05 μmole/ml. This is too close to the inherent error of the assay procedure to preclude a small conversion of indoleglycerol to indoleglycerol phosphate. It should be noted that the stimulatory effect of indoleglycerol is significant only in *tr-3* strains, which cannot convert indoleglycerol phosphate to tryptophan. Also, indoleglycerol will support the slow growth of strains other than *tr-3* strains (*unpublished data*). Thus, it appears likely that the effective molecule is indoleglycerol phosphate and that it can act as a potent inducer of the formation of TS activity.

TABLE 8. *Effect of indole-3-glycerol on the formation of TS and PRAT activities by strain RC-33-1A (tr-1,3) grown in still culture*

Culture				Specific activity[a]	
L-Tryptophan (μmole/ml)	Indoleglycerol added (μmole/ml)	Dry wt (mg/ml)	Indoleglycerol recovered (μmole/ml)	TS	PRAT
0.15	0	1.0	<0.01	4.17	2.33
0.15	0.25	1.0	0.26	6.33	2.33
0.15	0.5	1.0	0.52	7.80	2.33
0.15	1.0	1.1	1.0	9.00	2.50
0.15	2.0	1.2	1.9	11.0	2.50
0.6	0	2.7	<0.01	1.83	0.50
0.6	0.25	2.6	0.25	4.83	0.67
0.6	0.5	2.6	0.50	6.17	0.50
0.6	1.0	2.6	0.95	8.00	0.50
0.6	2.0	2.4	1.9	8.83	0.67

[a] Specific activity values are nanomoles per minute per milligram of protein.

DISCUSSION

The experiments described indicate that the enzymes involved in tryptophan biosynthesis are not formed coordinately. This conclusion is not unexpected, in view of the fact that coordinate repression and derepression usually has been observed with enzymes that are determined by closely linked genes (e.g., 1, 22). The genetic loci specifying the enzymes involved in tryptophan biosynthesis in *Neurospora* are found in different linkage groups (3). The disparity in the relative rates of formation of AAS and InGPS activities (Fig. 1) is of particular interest, since these enzymes are usually found in a molecular aggregate (11) and AAS activity appears to be dependent on the association of the *tr-2* gene product with that of the *tr-1* gene product which, alone, exhibits InGPS activity (10, 13). It might be proposed that the cells initially contained a relative excess of *tr-1* gene product (InGPS) with which the newly formed *tr-2* gene product could combine to form active AAS. It has been observed (D. C. Levin, Thesis, Reed College, 1966) that sucrose gradient sedimentation profiles of extracts from cells grown with repressing levels of tryptophan show a distinct, slower-moving shoulder of InGPS activity adjacent to a common peak of AAS and InGPS activities; after 8 hr of culture on limiting tryptophan, the shoulder is not apparant. These observations indicate that the *tr-1* and *tr-2* loci are not coordinately controlled and that the aggregation of *tr-1* and *tr-2* gene products occurs after their separate syntheses, rather than being requisitely produced as an aggregate. The latter conclusion has also been derived from in vitro complementation studies (2) with *tr-1* and *tr-2* mutants.

The effects of amitrole (histidine depletion, imidazoleglycerol phosphate, or both) and indoleglycerol phosphate indicate that the formation of TS activity is disproportionately stimulated by these compounds. Again, this shows that the formation of tryptophan biosynthetic enzymes is not requisitely coordinate. However, indoleglycerol phosphate and amitrole do appear to positively influence the formation of all those enzymes to some degree. It is noteworthy that the

effects of tryptophan depletion, amitrole, and indoleglycerol phosphate do not appear to be additive.

Several facets of tryptophan regulation reported here in experiments and in cited literature may be summarized. The derepression of tryptophan enzymes is effected by a limitation of tryptophan or of histidine and by indoleglycerol phosphate and imidazoleglycerol phosphate. The effects of tryptophan limitation are not reversed by histidine; the effects of a limitation of histidine are not reversed by tryptophan. Neither the indoleglycerol phosphate nor the imidazoleglycerol phosphate actions are significantly altered by tryptophan or histidine. The non-additivity of these individual effects on the formation of tryptophan enzymes and their relatively independent action tend to suggest that they are all involved in or with a single control unit. This unit might have a wider role, since it has been noted (5) that histidine deprivation also stimulates the formation of two arginine biosynthetic enzymes, and tryptophan deprivation enhances the formation of three histidine biosynthetic enzymes as well as the same two arginine enzymes.

It is of some interest to devise a speculative model which could account for these many and varied observations. First, it may be noted that the effect of histidine deprivation might be attributed to a lowering of histidinyl-tRNA (M. Carsiotis and G. Lester, Bacteriol. Proc., p. 136, 1968). This suggests a role for activated histidine in the repression of tryptophan enzymes, and, in view of the above observations, perhaps of histidine and arginine enzymes as well. It might be supposed that similar roles may be played by tryptophanyl- and argininyl-tRNA. The proposed active repressing unit would require the presence of all three aminoacyl-tRNA species for its action, and an insufficiency of any one component would render the unit ineffective and a derepression of enzyme formation would occur. Increasing the levels of the remaining components would have no effect, nor would the deprivation of a second component have an additive effect. The same unit would affect the formation of other enzymes, and, here too, its effectiveness in repressing the formation of these enzymes would be dependent on its total integrity. The multifaceted nature of this unit might warrant its designation as a "polyrepressor." Such a unit would be consonant with the non-additive nature of the effects described above. It could explain the effects of imidazoleglycerol phosphate or indoleglycerol phosphate by assuming that these compounds might inhibit the charging of histidine and tryptophan tRNA or interfere with the association of aminoacyl-tRNA in a polyrepressor; in either case, an incomplete, multiply-ineffective unit would result. The molecular nature of the polyrepressor could be one of an association of aminoacyl-tRNA species with a special protein (a histone?), or just with each other; it could have the potential of operating at a transcriptional or at a translational level. The metabolic aspects of this model may seem analogous to those of multivalent repression systems in bacteria (25). The major difference is that multivalent repression involves effector end products which are usually derived from closely related metabolic pathways, whereas the end products considered above have quite diverse origins. In any case, the validity of the polyrepressor model is only its present consistency with observed phenomena; its usefulness may be in its refutation.

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LITERATURE CITED

1. Ames, B. N., and B. Garry. 1959. Coordinate repression of the synthesis of four histidine biosynthetic enzymes by histidine. Proc. Nat. Acad. Sci. U.S.A. **45**:1453–1461.
2. Arroyo-Begovich, A., and J. A. DeMoss. 1969. In vitro formation of an active multienzyme complex in the tryptophan pathway of *Neurospora crassa*. Proc. Nat. Acad. Sci. U.S.A. **64**:1072–1078.
3. Barratt, R. W., D. Newmeyer, D. D. Perkins, and L. Garnjobst. 1954. Map construction in *Neurospora crassa*. Advan. Genet. **6**:1–93.
4. Bonner, D. M., C. Yanofsky, and C. W. H. Partridge. 1952. Incomplete genetic blocks in biochemical mutants of *Neurospora*. Proc. Nat. Acad. Sci. U.S.A. **38**:25–34.
5. Carsiotis, M., R. F. Jones, A. M. Lacy, T. J. Cleary, and D. B. Fankhauser. 1970. Histidine-mediated control of tryptophan biosynthetic enzymes in *Neurospora crassa*. J. Bacteriol. **104**:98–106.
6. Carsiotis, M., and A. M. Lacy. 1965. Increased activity of tryptophan biosynthetic enzymes in *Neurospora crassa*. J. Bacteriol. **89**:1472–1477.
7. Crawford, I. P., and I. C. Gunsalus. 1966. Inducibility of tryptophan synthetase in *Pseudomonas putida*. Proc. Nat. Acad. Sci. U.S.A. **56**:717–724.
8. DeMoss, J. A. 1965. The conversion of shikimic acid to anthranilic acid by extracts of *Neurospora crassa*. J. Biol. Chem. **240**:1231–1235.
9. DeMoss, J. A., and D. M. Bonner. 1959. Studies on normal and genetically altered tryptophan synthetase from *Neurospora crassa*. Proc. Nat. Acad. Sci. U.S.A. **45**:1405–1412.
10. DeMoss, J. A., R. W. Jackson, and J. H. Chalmers, Jr. 1967. Genetic control of the structure and activity of an enzyme aggregate in the tryptophan pathway of *Neurospora crassa*. Genetics **56**:413–424.
11. DeMoss, J. A., and J. Wegman. 1965. An enzyme aggregate in the tryptophan pathway of *Neurospora crassa*. Proc. Nat. Acad. Sci. U.S.A. **54**:241–247.
12. Frank, L. H., and R. D. DeMoss. 1957. Specific enzymic method for the estimation of L-tryptophan. Arch.

Biochem. Biophys. **67**:387-397.
13. Gaertner, F. H., and J. A. DeMoss. 1969. Purification and characterization of a multienzyme complex in the tryptophan pathway of *Neurospora crassa*. J. Biol. Chem. **244**:2716-2725.
14. Gibson, F. 1964. Chorismic acid: purification and some chemical and physical studies. Biochem. J. **90**:256-261.
15. Gordon, S. A., and R. S. Weber. 1951. Colorimetric estimation of indoleacetic acid. Plant Physiol. **26**:192-195.
16. Lester, G. 1961. Some aspects of tryptophan synthetase formation in *Neurospora crassa*. J. Bacteriol. **81**:964-973.
17. Lester, G. 1963. Regulation of early reactions in the biosynthesis of tryptophan in *Neurospora crassa*. J. Bacteriol. **85**:468-475.
18. Lester, G. 1968. In vivo regulation of intermediate reactions in the pathway of tryptophan biosynthesis in *Neurospora crassa*. J. Bacteriol. **96**:1768-1773.
19. 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.
20. Matchett, W. H., and J. A. DeMoss. 1962. Factors affecting increased production of tryptophan synthetase by a TD mutant of *Neurospora crassa*. J. Bacteriol. **83**: 1294-1300.
21. Matchett, W. H., and J. A. DeMoss. 1963. Direct evidence for a tryptophan-anthranilic acid cycle in *Neurospora*. Biochim. Biophys. Acta **71**:632-642.
22. Matsushiro, A., K. Sato, J. Ito, S. Kida, and F. Imamoto. 1965. On the transcription of the tryptophan operon in *Eschericia coli*. J. Mol. Biol. **11**:54-63.
23. Smith, O. H., and C. Yanofsky. 1960. 1-(*O*-Carboxyphenylamino)-1-deoxyribulose 5-phosphate, a new intermediate in the biosynthesis of tryptophan. J. Biol. Chem. **235**:2051-2057.
24. Turner, J. R., and W. H. Matchett. 1968. Alteration of tryptophan-mediated regulation in *Neurospora crassa* by indoleglycerol phosphate. J. Bacteriol. **95**:1608-1614.
25. Umbarger, H. E. 1969. Regulation of amino acid metabolism. Annu. Rev. Biochem. **38**:323-370.
26. Vogel, H. J. 1964. Distribution of lysine among fungi: evolutionary implications. Amer. Natur. **98**:435-446.
27. Vogel, H. J., and D. M. Bonner. 1956. Acetyl-ornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97-106.
28. Wegman, J., and J. A. DeMoss. 1965. The enzymatic conversion of anthranilate to indoleglycerol phosphate in *Neurospora crassa*. J. Biol. Chem. **240**:3781-3788.
29. Yanofsky, C. 1955. Tryptophan synthetase from *Neurospora*, p. 233-238. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 2. Academic Press Inc., New York.
30. Yanofsky, C. 1956. The enzymatic conversion of anthranilic acid to indole. J. Biol. Chem. **223**:171-184.
31. Yanofsky, C., and E. S. Lennox. 1959. Transduction and recombination study of linkage relationships among genes controlling tryptophan synthesis in *Escherichia coli*. Virology **8**:425-447.