

# Alteration of Tryptophan-mediated Regulation in *Neurospora crassa* by Indoleglycerol Phosphate

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The accumulation of imidazoglycerol phosphate during growth of *Neurospora crassa* in the presence of 3-amino-1,2,4-triazole was found to cause derepression of tryptophan synthetase and to inhibit the induction of kynureninase. Accumulation of indoleglycerol phosphate in response to growth in the presence of indole acrylic acid or anthranilic acid was also accompanied by derepressed synthesis of tryptophan synthetase. Enzyme synthesis in mutants (*his-7* and *trp-4*) unable to form these intermediates was not altered under similar conditions. The rate of formation of tryptophan synthetase and kynureninase was found to differ in the presence of tryptophan and indole.

The tryptophan biosynthetic pathway in *Neurospora crassa* has been studied in many laboratories. As a result of these investigations, a reasonably complete picture of the number of enzymes involved and the number and nature of the genetic loci concerned with the production of these enzymes is available (1, 3, 7, 8; J. Wegman, Ph.D. Thesis, Univ. of California, San Diego, 1964). The metabolic regulation of this system is, however, less well understood. Previous studies have shown that tryptophan inhibits the activity of the first enzyme of the pathway, anthranilate synthetase (14), by feedback inhibition (7). Other evidence shows that tryptophan can repress the formation of the terminal enzyme, tryptophan synthetase (13, 16), and can induce the formation of kynureninase, an enzyme in the tryptophan-degradative pathway which converts formyl kynurenine to formyl anthranilic acid (12, 23). Lester (13) demonstrated that conidia of tryptophan auxotrophs germinated in the absence of tryptophan had two to three times higher levels of tryptophan synthetase than conidia germinated in the presence of tryptophan, and that the wild-type strain was always fully repressed, presumably by endogenously synthesized tryptophan.

Matchett and DeMoss (16) also found it impossible to derepress wild-type *Neurospora*, and, of the tryptophan auxotrophs they examined, only strain td-201 (lacking reaction 1 and 3 of tryptophan synthetase) was derepressible. In addition, they obtained evidence indicating the presence of two distinct pools of tryptophan in *Neurospora*, and established that endogenously synthesized tryptophan was preferentially channeled to protein while exogenously supplied tryptophan was

preferentially channeled into the tryptophan-degradative pathway. On the basis of these results, they suggested that the larger or expandable pool could be responsible for control of the cycling enzymes, and that the smaller metabolic pool could function in the regulation of biosynthetic enzymes.

An investigation of tryptophan regulation in *Escherichia coli*, by Lester and Yanofsky (15), showed that metabolic intermediates could elicit control. These investigators found that anthranilic acid stimulated the formation of tryptophan synthetase, presumably by inhibiting the synthesis of indoleglycerol phosphate and consequently lowering the level of intracellular tryptophan.

In this investigation we attempted to elucidate the role of tryptophan as an effector in control mechanisms in *Neurospora*. These studies concern: (i) the role of biosynthetic intermediates in the regulatory processes, (ii) the possibility that free tryptophan is not the active regulatory effector, and (iii) the basis of the apparent difference in regulation found between internally formed tryptophan and externally supplied tryptophan.

We have found that the conditions which cause the accumulation of indoleglycerol phosphate or structurally related imidazole compounds in *Neurospora*, even in the presence of tryptophan, are accompanied by derepression of tryptophan synthetase. The tryptophan-mediated induction of kynureninase is also inhibited under some of these conditions. An interpretation of these results is that tryptophan may be converted to a derivative before it can function in the regulatory mechanisms, and that indoleglycerol phosphate inhibits the formation of this derivative. The regulation of

these two enzymes is also sensitive to the source of tryptophan; externally supplied tryptophan is a more effective inducer of kynureninase and internally formed tryptophan is a more effective repressor of tryptophan synthetase. The regulatory response to the origin of tryptophan is apparently related to the pool distribution of tryptophan. The nature of this relationship is, however, not well understood.

#### MATERIALS AND METHODS

*Neurospora strains and medium.* The auxotrophic strains of *N. crassa* used and their enzymatic blocks were: *trp-3* (strain td-201, a mutant which is deficient in reactions 1 and 3 of tryptophan synthetase but which retains the ability to catalyze the condensation of indole and serine to form tryptophan), *trp-4* (strain 32092, a mutant which lacks PR-transferase), *his-7* (strain 458, lacking isomerase); wild-type (strain 74A) was also used. All strains were maintained on solid Vogel's minimal medium and were transferred every 30 days. L-Tryptophan (150  $\mu\text{g}/\text{ml}$ ) was supplied for growth of tryptophan auxotrophs and L-histidine (350  $\mu\text{g}/\text{ml}$ ) was supplied for growth of histidine auxotrophs. Conidia were prepared from cultures grown on solid minimal medium with the appropriate supplement for auxotrophs. These cultures were inoculated from conidial stocks, incubated for 3 days at 30 C, and then placed at 25 C under illumination for 2 to 3 days for conidiation. These cultures were used within 12 days after inoculation.

*Preparation of experimental mycelia.* Mycelial suspensions were prepared by inoculation of 1 liter of liquid minimal medium, containing appropriate supplements for auxotrophs, with conidia from an agar flask culture to a final concentration of  $5 \times 10^6$  conidia per ml. The conidia were harvested from an agar culture (60 ml of solid medium in a 250-ml flask) by suspending with sterile distilled water and filtering through cheesecloth. The liquid culture was incubated on a rotary shaker at 30 C for 16 hr, harvested by filtration, washed with distilled water, and resuspended in fresh minimal medium to a concentration of 0.4 mg (dry weight) per ml. All experimental additions were made to this final suspension.

*Sampling and preparation of cell extracts.* The final mycelial suspension with added supplements was incubated in a rotary shaker at 30 C. Samples (100 ml) were removed at 1-hr intervals, harvested on filter paper, washed with distilled water, frozen, and lyophilized. The lyophilized mycelia were weighed and powdered by the use of a Vortex Junior mixer, as described by Carsiotis and Lacy (4). The powder was extracted with potassium phosphate buffer (0.1 M, pH 7.8) at 4 C for 30 min. The extract was clarified by centrifugation at  $12,000 \times g$  for 15 min and was stored at -20 C. By use of Sorvall heavy-wall glass centrifuge tubes (12 ml) and the Sorvall SS-34 rotor, samples (50 to 150 mg) of dry mycelia could be powdered, extracted, and centrifuged in the same tube. Enzyme assays and determinations of intracellular compounds were performed with the crude extract.

*Measurement of tryptophan synthetase and kynu-*

*reninase.* Tryptophan synthetase was measured, with a sample of the crude extract, by the disappearance of indole according to the procedure of Yanofsky (24). One unit of activity is defined as that amount of enzyme required to cause the disappearance of 1  $\mu\text{mole}$  of indole per hour at 37 C. Kynureninase was assayed by a modification of the procedure of Jakoby and Bonner (12). The reaction mixture contained 0.03 to 0.06 ml (0.4 to 0.9 mg of protein) of the crude extract, 1  $\mu\text{mole}$  of L-kynurenine sulfate, 3  $\mu\text{moles}$  of  $\text{MgSO}_4$ , 50  $\mu\text{moles}$  of tris(hydroxymethyl)amino-methane buffer (pH 8.0), 0.16  $\mu\text{mole}$  of pyridoxal phosphate, and water to a total volume of 1.2 ml. The reaction mixture was incubated for 15 min at 37 C and was stopped by the addition of 0.2 ml of 1 M  $\text{HClO}_4$ . An identical reaction mixture was prepared for each sample to which 0.2 ml of 1 M  $\text{HClO}_4$  was added at zero-time. After the addition of  $\text{HClO}_4$ , the tubes were incubated at 4 C for 30 min to facilitate protein precipitation and then were centrifuged. A portion of the supernatant fluid (0.5 ml) was diluted to 5 ml with potassium phosphate buffer (0.15 M, pH 5.5). The fluorescence of the anthranilic acid produced was measured on a Turner fluorometer. One unit equals that amount of enzyme required to form 1  $\mu\text{mole}$  of anthranilic acid per hour at 37 C.

*Other assay methods.* Indoleglycerol (phosphate) was measured in extracts as toluene-extractable indole after alkaline hydrolysis at 100 C. This assay, when performed on cell extracts, was assumed to measure indoleglycerol phosphate. Imidazoglycerol phosphate was measured in extracts by periodate oxidation followed by alkaline *n*-butyl alcohol extraction (2). Indole was measured colorimetrically (23); tryptophan in extracts was assayed with tryptophanase (6).

*Reagents and chemicals.* The herbicide 3-amino-1,2,4-triazole was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. L-Tryptophan, indole, anthranilic acid, and indole derivatives were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

#### RESULTS

The results of Carsiotis and Lacy (4, *personal communication*) and Jones and Carsiotis (*Bacteriol. Proc.*, p. 114, 1967) with histidine mutants which were derepressible for tryptophan synthetase and the work of Crawford and Gunsalus (5) with derepressible mutants of *Pseudomonas putida* suggested to us that biosynthetic intermediates of tryptophan and structurally related imidazole compounds could be important in the control mechanisms of enzymes related to tryptophan metabolism in *Neurospora*. Figure 1 shows the effects of two of these compounds on the formation of tryptophan synthetase (a) and kynureninase (b) in the tryptophan auxotroph, strain td-201. Derepression of tryptophan synthetase occurred in the presence of tryptophan and 3-amino-1,2,4-triazole or anthranilic acid, with the concomitant accumulation (c) of imidazoglycerol phosphate and indoleglycerol phosphate,

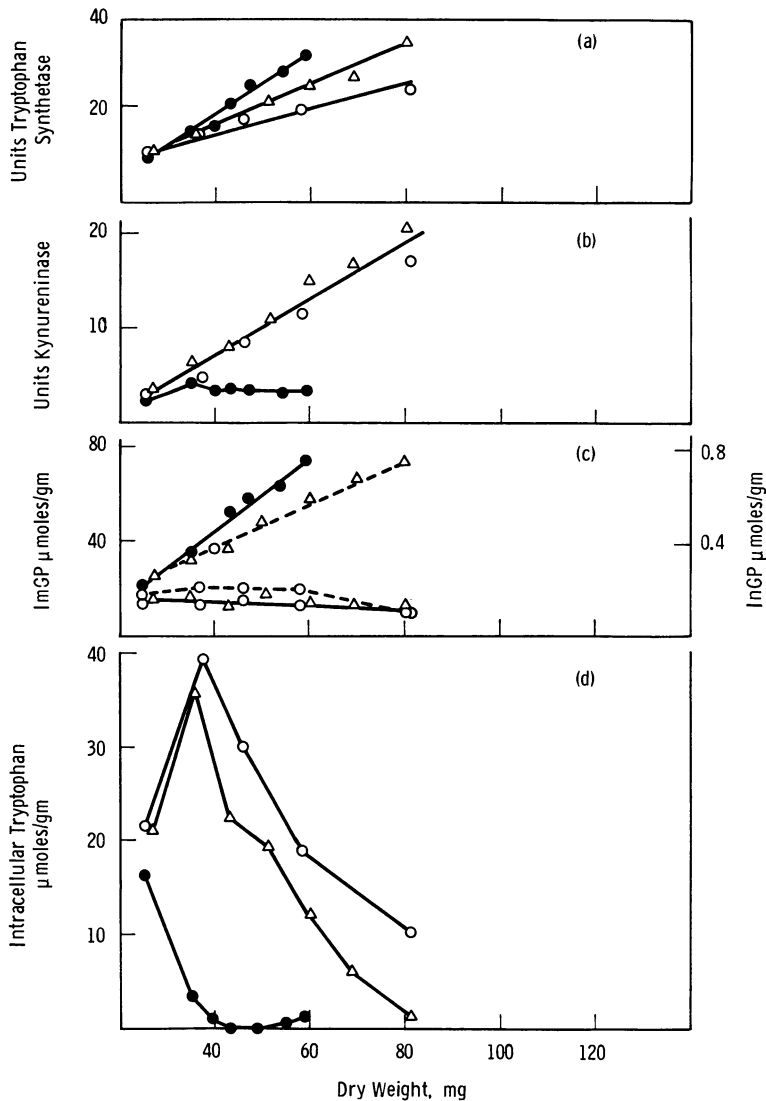


Fig. 1. Differential rates of enzyme synthesis and accumulation of intermediates by strain *td-201* in response to 3-amino-1,2,4-triazole (3AT) and anthranilic acid (AA). Initial concentration of 3AT, 5  $\mu$ moles/ml; initial concentration of AA 2.19  $\mu$ moles/ml; initial concentration of tryptophan 0.75  $\mu$ mole/ml. Cultural conditions: tryptophan,  $\circ$ ; 3AT + tryptophan,  $\bullet$ ; AA + tryptophan,  $\triangle$ . Abbreviations: ImGP, imidazoleglycerol phosphate (solid line); InGP, indoleglycerol phosphate (dashed line).

respectively. Imidazoleglycerol phosphate is an intermediate of histidine which accumulates as a result of inhibition of imidazoleglycerol phosphate dehydratase by 3-amino-1,2,4-triazole (11). The presence of 3-amino-1,2,4-triazole completely inhibited the tryptophan-mediated induction of kynureninase (b). At the concentration of indoleglycerol phosphate accumulated in the presence of anthranilic acid, however, the induction of kynureninase was not affected. Figure 1d shows that in-

tracellular tryptophan was not reduced by these treatments to values which would allow derepression (16) or which would be insufficient to induce kynureninase. Although 3-amino-1,2,4-triazole had an inhibitory effect on either the uptake of tryptophan or the maintenance of intracellular pools of tryptophan, the effect of this compound on regulation of tryptophan synthetase and kynureninase does not appear to depend on this inhibition. Derepression of tryptophan synthetase and in-

hibition of kynureninase induction were apparent before the intracellular tryptophan levels were markedly reduced. Figure 2 shows the results of a similar set of experiments with the wild-type strain 74A. Anthranilic acid or 3-amino-1,2,4-triazole in the presence of tryptophan caused derepression of tryptophan synthetase (a), and 3-amino-1,2,4-triazole inhibited induction of kynureninase (b). Since the wild type is capable of converting indoleglycerol phosphate to tryptophan, this intermediate was not accumulated in measurable amounts as it was in the mutant td-201. Inhibition of the ability of tryptophan to elicit control under these conditions was not due to a decreased transport of tryptophan, since these aberrations can be produced when indole is used as a source of tryptophan.

Several derivatives of indole were tested for their ability to cause derepression (Fig. 3). Of the six compounds tested, only indole acrylic acid elicited derepression of tryptophan synthetase; all others gave normal repressed levels of enzyme. Further, only indole acrylic acid caused the accumulation of indoleglycerol phosphate, an effect which appears to result from inhibition of the conversion of indoleglycerol phosphate to tryptophan. In contrast, the increased level of indoleglycerol phosphate resulting from an exogenous supply of anthranilic acid appears to result simply from increasing the supply of its precursor.

It appears that the activity of indole acrylic acid in the tryptophan pathway is analogous to the activity of 3-amino-1,2,4-triazole in the histidine pathway in that indole acrylic acid causes indoleglycerol phosphate to accumulate by inhibiting its conversion to tryptophan, while 3-amino-1,2,4-triazole causes imidazoleglycerol phosphate to accumulate by inhibiting its conversion to imidazoleacetol phosphate.

Regardless of the mechanisms involved, the accumulation in cells of either indoleglycerol phosphate or imidazoleglycerol phosphate appears to interfere with the regulatory action of tryptophan. This conclusion is further supported by studies with mutants. The mutant 32092 lacks the enzyme PR-transferase and is therefore unable to form indoleglycerol phosphate from anthranilic acid. Similarly, *his-7* mutants (e.g., strain 458) are blocked in the histidine pathway prior to imidazoleglycerol phosphate and do not accumulate this intermediate. Figure 4b shows the relative rate of formation of tryptophan synthetase by *trp-4* mutant 32092 in the presence of indole acrylic acid, anthranilic acid, and 3-amino-1,2,4-triazole. Only 3-amino-1,2,4-triazole could elicit derepression of tryptophan synthetase in this mutant. Neither indole acrylic acid nor anthra-

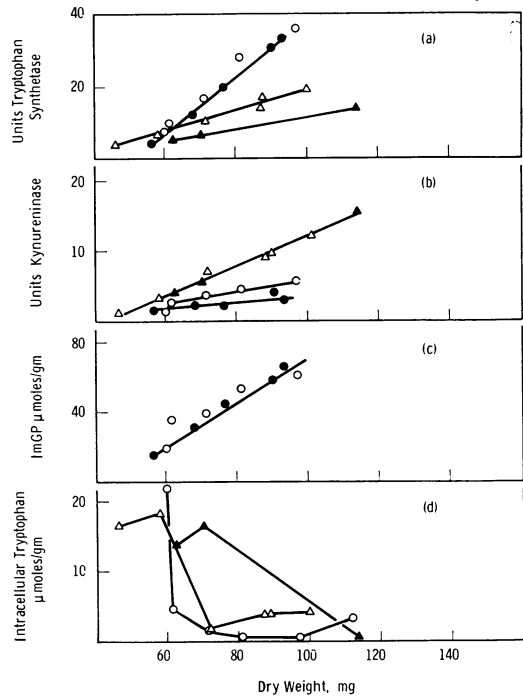


FIG. 2. Differential rates of enzyme synthesis and accumulation of intermediates by wild-type strain 74A in response to 3-amino-1,2,4-triazole (3AT) and anthranilic acid (AA). Initial concentration of cultural additions was the same as in Fig. 1. Cultural conditions: 3AT + tryptophan  $\circ$ ; 3AT,  $\bullet$ ; AA + tryptophan,  $\triangle$ ; tryptophan,  $\blacktriangle$ .

nilic acid could derepress tryptophan synthetase. Indoleglycerol phosphate, if present, was below detectable levels. Figure 4a shows that, although indole acrylic acid and anthranilic acid cause the *his-7* mutant to form tryptophan synthetase at an increased rate, 3-amino-1,2,4-triazole is without effect. In this case, indoleglycerol phosphate was accumulated in response to indole acrylic acid or anthranilic acid, but imidazoleglycerol phosphate was not observed in response to 3-amino-1,2,4-triazole. These experiments show that the compounds anthranilic acid, indole acrylic acid, and 3-amino-1,2,4-triazole are themselves without direct effect and suggest that the accumulated intermediates are the active compounds.

In all cases where different relative rates of enzyme synthesis were observed, mixing experiments were performed with the extracts from all conditions, and neither activation nor inhibition of activity could be detected.

Matchett and DeMoss (18) demonstrated that *Neurospora* forms two distinct pools of tryptophan, a metabolic pool and an expandable pool.

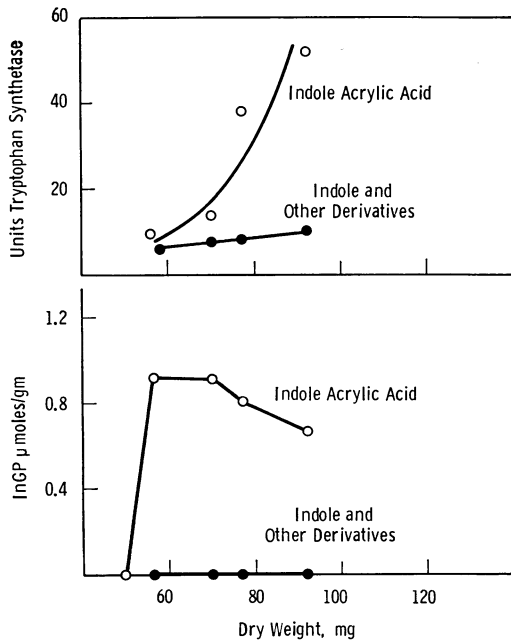


FIG. 3. Effect of indole derivatives on tryptophan synthetase and indoleglycerol phosphate (InGP) accumulation by wild-type strain 74A. Initial concentration of indole and all derivatives was  $0.5 \mu\text{mole/ml}$ . Other derivatives tested were indole acetic acid, indole propionic acid, indole butyric acid, indole lactic acid, and indole pyruvic acid.

The smaller of the two, the metabolic pool, is the source of tryptophan for protein synthesis; therefore, either externally supplied or internally synthesized tryptophan must enter this pool before being incorporated into protein. Indole enters the cells and is converted to tryptophan instantly, without formation of an indole pool. Tryptophan of biosynthetic origin preferentially populates the metabolic pool. Externally supplied tryptophan, although capable of entering either pool, preferentially enters the larger expandable pool and is degraded to anthranilic acid via the tryptophan-degradative pathway (17). Thus, when  $^{14}\text{C}$ -indole and unlabeled tryptophan were supplied to growing mycelia of *Neurospora*, the specific activity of tryptophan in protein was higher than tryptophan in the acid-extractable portion of the cell sap. Further, the products of degradation of tryptophan were either equal to or lower than the specific radioactivity of the acid-soluble tryptophan.

Figure 5 shows relative rates of the formation of tryptophan synthetase and kynureninase in the presence of tryptophan and indole. Tryptophan of biosynthetic origin is a more effective repressor of tryptophan synthetase (a) and a less effective

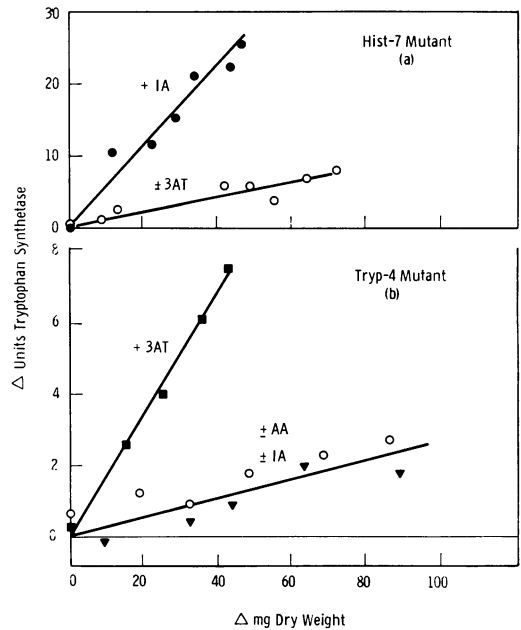


FIG. 4. Differential rates of tryptophan synthetase formation by a *his-7* mutant (a) a *trp-4* mutant (b) in response to indole acrylic acid (IA;  $0.5 \mu\text{mole/ml}$ ), anthranilic acid (AA;  $2.19 \mu\text{moles/ml}$ ), and 3-amino-1,2,4-triazole (3AT;  $5 \mu\text{moles/ml}$ ). The *his-7* mutant (a) was grown in minimal medium plus additions; the *trp-4* mutant (b) was grown in minimal medium containing  $0.75 \mu\text{mole/ml}$  of L-tryptophan plus additions.

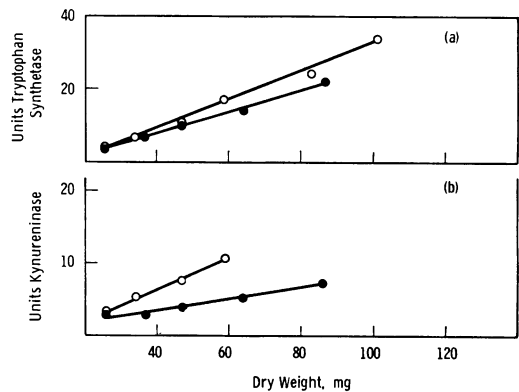


FIG. 5. Differential rates of formation of tryptophan synthetase and kynureninase by a *trp-3* mutant (*td-201*). Initial concentration of indole and tryptophan was  $0.5 \mu\text{mole/ml}$ . Cultural conditions: indole, ●; tryptophan, ○.

inducer of kynureninase (b) than is tryptophan supplied to the cells. Since, in the presence of either tryptophan or indole, the biosynthetic pathway is completely blocked by feedback in-

hibition of anthranilate synthetase, there are no biosynthetic intermediates accumulated by the cells. The differential ability of internally synthesized tryptophan and externally supplied tryptophan to control the formation of tryptophan synthetase and kynureninase may be related to the pool distribution (18) of tryptophan. The differential ability of internal and external tryptophan to enter in regulatory processes is apparent not only when these tryptophan sources are examined alone but also in the presence of 3-amino-1,2,4-triazole and anthranilic acid. In the presence of 3-amino-1,2,4-triazole or anthranilic acid, the differential rate of formation of tryptophan synthetase is greater in the presence of tryptophan than in the presence of indole (Fig. 6). Therefore, although these compounds can be used to derepress tryptophan synthetase, they probably do not alter the distribution of tryptophan between the two pools.

Although the differences in relative rate of enzyme synthesis between cultures containing tryptophan and indole are small, they are completely reproducible, and this effect can be shown in both the wild type (strain 74A) and the *trp-3* mutant (strain td-201).

#### DISCUSSION

Evidence has been presented recently concerning the role that aminoacyl-soluble ribonucleic acid (sRNA) derivatives play in the regulation of repressible enzyme systems. Schlesinger and Magasanik (21) demonstrated that the histidine

analogue  $\alpha$ -methylhistidine derepresses the histidine pathway of *E. coli*. It was proposed that the derepressive action of the analogue is due to inhibition of attachment of histidine to sRNA, implicating histidinyl-sRNA as the repressor. Eidlic and Neidhardt (9) have shown that *E. coli* mutants possessing a temperature-sensitive valyl-sRNA synthetase are derepressed for valine-controlled enzymes when shifted to the nonpermissive temperature, and that the most probable cause for the derepression is the decreased ability of the cells to form valyl-sRNA (19), rather than the inability to activate the amino acid. A report by Freundlich (10) supports this view. He demonstrated that the valine analogue  $\alpha$ -amino- $\beta$ -chlorobutyric acid, which can replace valine in repression of valine-isoleucine enzymes, can protect sRNA<sub>val</sub> against periodate oxidation, whereas  $\alpha$ -amino-butyric acid can neither repress these enzymes nor protect sRNA<sub>val</sub> against periodate oxidation. Since both of the analogues can be activated by valyl-sRNA synthetase, it is apparent that attachment of the amino acid to sRNA, rather than activation by the sRNA synthetase, is the critical reaction in repressor formation. Histidine regulatory mutants of *Salmonella typhimurium* selected for resistance to thiazole-alanine (20) were found to fall into four distinct classes, two of which have some degree of impaired ability to synthesize histidinyl-sRNA, and all of which were unable to repress fully the histidine operon.

The results presented here lead to the conclusion that, in *Neurospora*, tryptophan has the attributes generally ascribed to a small molecule effector, as that term is used in the context of the operon theory (22). Its action as an effector may be judged by two criteria: repression of tryptophan synthetase and induction of kynureninase. Indole and imidazole derivatives, accumulated in response to compounds which were shown to be inert, seem to interfere with the activity of tryptophan as an effector, since the effects of these compounds (indoleglycerol phosphate and imidazoleglycerol phosphate) occur in the presence of normally active concentrations of tryptophan. For this reason, it is attractive to suppose that these compounds inhibit the conversion of tryptophan to a form which is active in regulation of protein synthesis. The recent results implicating aminoacyl-sRNA derivatives in control suggest that, in our system, tryptophanyl-sRNA may be the active compound and further that the indole and imidazole derivatives may interfere in some way with the formation of this compound.

The observation that biosynthesized tryptophan is a more efficient repressor of tryptophan

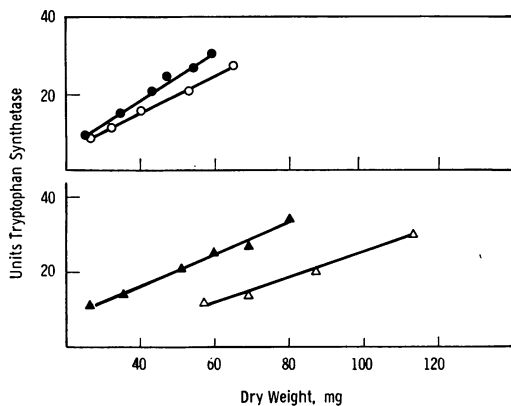


FIG. 6. Differential rates of formation of tryptophan synthetase in strain td-201; effect of 3-amino-1,2,4-triazole (3AT) and anthranilic acid (AA) on the source of tryptophan. Initial concentration of indole and tryptophan was 0.5  $\mu$ mole/ml. Initial concentration of 3AT and AA was the same as in Fig. 1. Cultural conditions: 3AT + tryptophan, ●; indole + 3AT, ○; tryptophan + AA, ▲; indole + AA, △.

synthetase and a less efficient inducer of kynureninase than is externally supplied tryptophan is, however, difficult to account for on the basis of a single effector derived from only one intracellular pool. It is apparent that endogenously formed tryptophan, which is preferentially used for protein synthesis, is also more available to the repression system and that exogenously supplied tryptophan is more readily available to the induction system.

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